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THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL] HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Mass. Special meetings of the Trustees shall be called by the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed. In addition, there shall be two groups of Trustees as follows:

(B) Trustees ex officio, who shall be the President and Vice President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer, and the Clerk:

(C) Trustees Emeriti, who shall be elected from *present* or *former* Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeritus* shall have all the rights of the Trustees except that Trustees *Emeritus* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation; they shall elect a President of the Corporation who shall also be Chairman of the Board of Trustees and who shall be elected for a term of five years and shall serve until his successor is selected and qualified; and shall also elect a Vice President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and who shall be elected for a term of five years and shall serve until his successor is selected and qualified; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

IV. REPORT OF THE DIRECTOR

To: The Trustees of the Marine Biological Laboratory
Gentlemen:

I submit herewith the report of the seventy-second session of the Marine Biological Laboratory.

During the past year the Laboratory made significant progress in rehabilitating some of its research space and facilities and also funds were obtained for a new research building and additional housing.

1. Policy

During the past several years there have been about thirty people including investigators and their co-workers engaged in research on a year-round basis at the Laboratory. Investigators have made application through the Laboratory to various granting agencies for support for their various projects. In general, such investigators have been provided with laboratory space and services for a limited tenure, usually no more than five years. In addition, there have been other investigators, either retired or on sabbatical leave, who have availed themselves of the opportunity to work at the Laboratory continuously for one or more years at the discretion of the Executive Committee.

There has been a growing interest among the members of the Corporation, particularly some of those engaged in invertebrate zoological research, in the possibility of developing year-round research programs at the Laboratory in marine systematics and ecology. It is felt by many that such programs will serve to strengthen the summer research programs and will represent the most profitable type of year-round research. The Board of Trustees concurred in a recommendation from the Executive Committee that such a combined systematics-ecology program be operated by the Laboratory, the staff to be selected by the Executive Committee. Efforts are being made to receive support for this program. Also, a grant has been obtained from the Office of Naval Research with which to explore the feasibility of developing a year-round research program in marine microbiology.

The Laboratory will continue to make research space and facilities available to retired scientists and those on sabbatical leave on the approval of the Executive Committee.

2. *Research Training Programs*

In 1959 the course in Physiology operated as a research training program under the direction of Dr. William McElroy with support from the National Institutes of Health. The success of this operation strongly supported the desirability of developing the other courses along similar lines. Research training program support has been obtained from the National Institutes of Health for Experimental Embryology starting in 1960 and for Marine Ecology starting in 1961. During this present winter the Old Lecture Hall has been completely remodeled to house the Experimental Embryology Training Program. Also in 1960 Invertebrate Zoology and Marine Botany will start operating as research training programs with support from the National Science Foundation. It is confidently anticipated that these programs will attract staff members of the same high caliber as have the courses in the past. At this writing the trainee applicants far exceed the numbers that can be accommodated in the various programs, which permits the selection of highly qualified individuals.

All the training programs will run for the entire summer season. Each program will operate in the manner best calculated in the minds of its director and his staff to develop background knowledge, technical competence and research interest in the trainees. It is anticipated that these programs will stimulate increased interest in marine biology since it will introduce the beginning investigator to marine research material early in his career and emphasize the unique advantages that such material presents for a great variety of problems.

3. *New Laboratory Building*

The current progress on the new building assures completion for 1960 summer occupancy barring unforeseen delays in the construction. The schedule is a very tight one so any interruption of the work will be most serious. Mr. Homer P. Smith, General Manager of the Laboratory, merits the highest commendation for the energetic and effective way in which he has promoted the construction of the building. The original planning called for completion of the building for 1961 summer occupancy so that the present schedule represents the gain of a full year.

4. *Grants, Contracts and Contributions*

The total income from these sources of support amounted to $181,726.00 in 1959. This represents 29.6% of the total income and is made up of the following accounts:

American Cancer Soc.—RC4A (+) Studies in Radiobiology	$ 3,300.00
AEC—1343—Program of Research on the Physiology of Marine Organisms Using Radioisotopes	16,165.00
NIH—4359—Biological Research on the Morphology, Ecology, Physiology, Biochemistry and Biophysics of Marine Organisms	40,000.00
NIH—5143—Training Program in Nerve Muscle Physiology	21,654.00

ONR—1497—Studies in Marine Biology	15,000.00
ONR—09701—Studies on Isolated Nerve Fibers	6,072.00
ONR—09702—Studies in Ecology	3,055.00
NSF—8295—Studies in Marine Biology	40,000.00
AEC–MBL–BM–78–59—Equipment	13,690.00
MBL Associates	4,170.00
Abbott Laboratories	1,000.00
Carter Products	1,000.00
Ciba Pharmaceutical Products, Inc.	1,000.00
Josephine B. Crane Foundation	2,000.00
Eli Lilly & Company	5,000.00
Merck Company Foundation	1,000.00
Schering Foundation, Inc.	1,000.00
Smith, Kline & French Foundation	3,000.00
The Upjohn Company	1,000.00
Wyeth Laboratories, Inc.	1,000.00
Olin Mathieson Chemical Corporation Charitable Trust	500.00
George Frederick Jewett Foundation	1,000.00
Miscellaneous Individual	120.00
	$181,726.00

5. *Future Plans*

During the summer of 1959 the Executive Committee in several meetings considered the immediate future needs of the Laboratory and recommended that steps be taken to (1) develop plans and obtain funds for a dormitory-dining hall building, (2) construct an additional 25 cottages on the Devil's Lane Property, and (3) prepare an application for funds to cover the detailed planning and construction of a research instruction building.

The concentration of both the research and dormitory buildings on our present campus creates a serious problem of congestion. The ten old wooden residences used as dormitories take up space out of proportion to the numbers they accommodate. Also they do not adapt satisfactorily to dormitory use. The present dining hall, constructed for table service, has been modified to cafeteria service but it leaves much to be desired in fully meeting the needs of the Laboratory. A new dormitory-dining hall facility is urgently needed in a convenient location off of our present limited campus. There were sixty applications in 1959 for the twenty-five new cottages built in the spring in the Devil's Lane Tract. An additional twenty-five cottages are urgently needed particularly since the training programs will attract a larger number of advanced students. A research training building to replace the wooden laboratories will provide modern facilities for the training program staffs and the trainees. Such a building will permit the consolidation under one roof of the variety of services which must be provided the present training programs housed in three wooden buildings. It will also permit certain economies in operation and maintenance.

Respectfully submitted,
PHILIP B. ARMSTRONG,
Director

MEMORIAL

Edmund Newton Harvey

by

Aurin M. Chase

The death of Edmund Newton Harvey from a heart attack on the morning of July 21st at his Penzance Point home was so unexpected as even now hardly to seem possible to his multitude of friends. Indeed, so difficult is it to realize that for a long time many of those who knew him will still expect to hear his voice in the corridors of the M.B.L., or to see him at their doors asking what's new, or reporting, with that characteristic enthusiasm, the most recent discovery in bioluminescence or in any one of a dozen other fields.

It is rare indeed for a man to reach the age ordinarily associated with retirement and still remain so young. Newton Harvey was equally at home with people of all ages, and they with him. He will be greatly missed at Woods Hole and the many other places where he lived, worked and played.

Having graduated from Germantown Academy and then received his Bachelor of Science at the University of Pennsylvania, Harvey went to Columbia for his graduate work and was awarded the Ph.D. in 1911. He started teaching immediately at Princeton where, virtually single-handed, he initiated courses in general physiology and biochemistry, subjects not often included in biology curricula at that time. In 1916 he married Ethel Nicholson Browne, herself a Columbia Ph.D. in zoology. They shared laboratories at Princeton and Woods Hole over the years, except for the period when it was necessary for her to devote full time to raising their two sons, Ned and Dick. The Harveys had a host of friends and greatly enjoyed interesting company. Many will remember, for example, the "Harvey Table" at the M.B.L. Mess as a center of good conversation and congeniality.

At Princeton, Newton Harvey was promoted to full professor in 1919 and, fourteen years later, became Henry Fairfield Osborn Professor of Biology, occupying that chair until his retirement in 1956. During his years of teaching he attracted many graduate students, most of whom based their Ph.D. theses on some aspect of bioluminescence. Nearly all his summers were spent at the M.B.L., where he had been elected a member of the Corporation in 1910, when barely out of college, and became a trustee in 1929. He served as Vice President of the Corporation from 1942 until 1952, and was always most active and influential in the affairs of the Laboratory. He was elected Trustee Emeritus in 1958. Although it was only natural that a man of his interests and energy should become involved in the direction and operation of many organizations—he was, for example, a trustee of the Bermuda Biological Station—the activities and welfare of the Marine Biological Laboratory were always closest to his heart.

With as many interests as he had, it is not surprising that Harvey belonged to some twenty societies covering the fields of physiology, biochemistry and biophysics, as well as less specialized areas of biology. He was a member of The National Academy of Sciences and The American Philosophical Society, and had been vice president and president of The International Society of Cell Biology. In 1953 he was president of The American Society of Zoologists, as he was also of The American Society of Naturalists in 1956. He was an associate editor of several journals, and served as Managing Editor of *The Journal of Cellular and Comparative Physiology* during the first sixteen years of its existence.

Among the formal awards made to him were the John Price Wetherill Medal of The Franklin Institute, in 1934, for his development of the centrifuge-microscope, and the Rumford Medal, by The American Academy of Arts and Sciences, in 1947, for his work in bioluminescence. He received the Certificate of Merit of the U. S. Armed Forces for his services, both experimental and advisory, during the second world war. More recently, the Johns Hopkins University awarded him an honorary Doctor of Science degree, and Temple University the honorary degree of Doctor of Laws.

Harvey's attitude toward research was always that of the explorer and pioneer. He opened up new regions for others to develop. His tremendous curiosity and drive were not satisfied by the sort of routine experimentation required to wrap up completely all the loose ends of a problem. Without his kind, the discovery of new things would be slow if it occurred at all. So extensively did he explore in the field of bioluminescence, for example, that it is difficult to do any experiment involving a luminous organism without finding, sooner or later, that Harvey had had the same idea and tested it—at least in a preliminary way—years before.

A person of such consuming curiosity could never be satisfied in a single line of research. His 250 or so published papers include such topics as cell permeability and oxidations, physical measurements at cell surfaces, brain potentials, effects of supersonic waves and of centrifugal forces on cells and—during the war—decompression sickness and wound ballistics. He loved instrumentation and was always eager to apply new apparatus and techniques to biological problems. His vast knowledge of organisms was most useful in this connection. But his greatest interest, and the one to which he returned again and again, and which was occupying him at the time of his death, was bioluminescence. It is for this that he is best known and the acknowledged authority throughout the world. He wrote four books on the subject, and was working on a fifth.

One who did not know Newton Harvey might get the mistaken impression from a review of his many scientific accomplishments that his time was spent entirely in the laboratory and the study. Nothing could be farther from the truth! He enjoyed life to the full! He was an excellent tennis player in his time and an experienced mountain climber, went on numerous scientific expeditions, and at one time was even reported to have been eaten by cannibals! At Woods Hole he always found time for swimming and sailing, and the Harveys' sloop, the "Rip Tide," carried many a happy party across Vineyard Sound to Edgartown, or down the islands to Tarpaulin Cove or Cuttyhunk.

He loved to surprise his friends in unusual and pleasant ways, such as pulling off the road into a secluded grove, opening the luggage compartment of his big Buick, and serving cocktails from the portable bar which nobody had suspected was there. Then would follow dinner at some nearby restaurant which he had previously tested and found to be good.

Yet in the midst of a social gathering his scientific interest might suddenly come to the fore. As when, once, he tossed a corked bottle to the ceiling so that all could observe that the large air bubble trapped inside did not rise in the bottle while it was in "free fall."

All those who have come in contact with Edmund Newton Harvey, his friends, colleagues, graduate students, and those fortunate undergraduates who did their senior theses under his direction, have absorbed some of his boundless enthusiasm and spirit. Certainly he will be sorely missed. But surely he would not have preferred to have gone otherwise than as he did; in full possession of all his faculties; vigorous, joyful and active until the last.

MEMORIAL

George H. A. Clowes

by

Philip B. Armstrong

Rarely do we encounter in one man such a variety of talents and interests as characterized George Henry Alexander Clowes. With unbounded energy, he used these talents effectively, following up his interests with persistent determination. He was a scientist and investigator who sparked many practical applications of basic research. He was a man of business, a civic leader, and patron of the fine arts and of music.

Dr. Clowes was born in Ipswich, England, in 1877, in a period of developing scientific interest. Through environment and natural bent, he early developed an interest in science. His family was in the business of producing intermediates for the brewing industry.

He attended the Royal College of Science in London from 1893–1896 and completed his studies for a Ph.D. degree in chemistry in 1899 at the University of Göttingen. His thesis was concerned with the methyl derivatives of sugar. After additional work in some of the other leading chemical laboratories in France and Germany, he came to this country in 1901 as chemist at the Institute for the Study of Malignant Diseases at Buffalo.

For the following period of fifteen years, Dr. Clowes published extensively, using a variety of approaches to the problem of cell division and growth, particularly as it applied to cancer. An important contribution concerned the evidence for immunity against cancer in mice after spontaneous remissions. Other papers dealt with the mechanism of the action of mustard gas, enzyme action in fermentation, ion antagonisms, and the action of anesthetics in biological and physical systems. His outstanding scientific contributions were on the effects of calcium or sodium at oil-water interfaces. Surface phenomena remained a life-long interest resulting in many practical developments.

Dr. Clowes left Buffalo in 1918 to serve in the Chemical Warfare Service where he continued his work on mustard gas, particularly its mechanism of action. In order to study its action under most favorable experimental conditions, he spent the summer of 1918 at the Marine Biological Laboratory, working with Robert Chambers and Ralph Lillie. Although Dr. Clowes had a highly practical turn of mind, he had a deep appreciation of the potential significance of basic research. The scientific intellectual atmosphere of the Marine Biological Laboratory was so attractive to Dr. Clowes that he spent most of the following 40 summers at Woods Hole. At the Laboratory he found what he considered ideal biological materials for many of the basic problems in which he was interested.

After the war he joined the research staff of Eli Lilly and Company and became Director of Research in 1921. Here he served very effectively in the development of the commercial production of several products, starting with insulin. His earlier interest in salt antagonisms and the effects of pH on proteins served him in good stead in the precipitation and purification of insulin. He also played a prominent role in the development of liver extract, protamine insulin and penicillin. From the Marine Biological Laboratory with collaborative workers Homer Smith, Maurice Krahl, Anna Keltch and others, Dr. Clowes published a series of papers on the possible control of mitosis by chemical agents.

In 1918 he was elected a member of the Corporation of the Marine Biological Laboratory, served three terms as a trustee, and was elected a Trustee Emeritus in 1948. He was active in seeking support for the Laboratory as well as contributing generously himself. In Indianapolis he played an active role in the development of the Indianapolis Symphony Orchestra and the John Huron Art Institute.

Dr. Clowes enjoyed a felicitous marriage. He is survived by his wife, Mrs. Edith Whitehill Clowes, who shared with him many of his civic and philanthropic interests. Also surviving him are two sons, Dr. G. H. A. Clowes, Jr. and Allen W. Clowes. Dr. Clowes died in Woods Hole on August 25, 1958. He will be remembered by his Woods Hole friends for his outstanding generosity and kindness and his active participation in the scientific and physical development of the Marine Biological Laboratory. He had a keen interest in the work of others, participated actively in the scientific discussion at the Laboratory, and was always ready to give help in the development of science.

MEMORIAL

Eugene Floyd Du Bois

by

Paul Reznikoff

Dr. Eugene Floyd Du Bois died on February 12, 1959, at his home at the age of 76. His death is not only a great loss to medicine and physiology but to his many students, house officers and colleagues who were associated with him at Cornell University Medical College, Bellevue Hospital, the New York Hospital and the Marine Biological Laboratory, it is a personal tragedy.

Doctor Du Bois was born on June 4, 1882 in West New Brighton, Staten Island, New York, the son of Eugene and Anna Brooks Du Bois. He attended the Staten Island Academy and Milton Academy in Massachusetts and received his A.B. degree from Harvard in 1903 and his M.D. degree from Columbia in 1906. In 1948 he was awarded an honorary Doctor of Science degree by Rochester University.

After interning at the Presbyterian Hospital (1907–08) and acting as assistant pathologist (1909) at this institution he decided to secure a training in bacteriology. But just before he was about to leave for France he changed his plans at the suggestion of Dr. John Howland and he went to Germany to study metabolism. Dr. Graham Lusk visited the laboratory in Berlin and met Dr. Du Bois there. This was the beginning of a lifelong association of these two pioneers in the field of metabolism. When Dr. Du Bois returned to the United States he soon became the Medical Director of the Russell Sage Institute of Pathology, of which Dr. Lusk was the Scientific Director. Thus Dr. Du Bois became a scientific descendant of Lavoisier. Under Du Bois' guidance the Russell Sage Institute has had world-wide influence in advancing scientific knowledge in the field of metabolism and from his "Calorimeter Room" there have gone forth many of our most important scientists and medical educators.

Doctor Du Bois was Director of the Second (Cornell) Medical Division of Bellevue Hospital from 1919 to 1932, Professor of Medicine at the Cornell University Medical College from 1930 to 1941, Physician-in-Chief at the New York Hospital from 1932 to 1941, and Professor of Physiology at Cornell from 1941 to 1950 when he became Emeritus Professor.

Some of Dr. Du Bois' most important contributions were concerned with his work for the United States Navy. Because of his modesty few of his associates knew that he was an outstanding authority in the fields of submarine warfare and aviation medicine. For heroism in the conduct of hazardous experiments during World War I he received the Navy Cross. During the second World War he was recognized by Commendation and Ribbon Bar. He retired from the Navy with the rank of Captain and continued to work with the military service until the very day of his death.

He belonged to many societies—the National Academy of Sciences, the Philosophical Society, the American Physiological Society, the Aero Medical Association, the Society for Experimental Biology and Medicine, and was president of the American Society for Clinical Investigation, of the Association of American Physicians, of the Harvey Society and of the Institute of Nutrition.

He became a member of the Corporation of the Marine Biological Laboratory in 1929 and was elected a Trustee in 1942. He was re-elected in 1944 for an additional full term to 1948. In 1952 he was elected Trustee Emeritus which position he held until his death. He was as conscientious in his duties to the Laboratory as he was in all undertakings and even during the later years of his incapacity he attended the meetings of the Trustees despite his physical limitations. He planned to be present at the Annual Winter Meeting which took place just one day after his death.

Many honors came to Dr. Du Bois, such as the Kober Medal of the Association of American Physicians in 1947 and the Academy Medal of the New York Academy of Medicine in 1956. He was to receive the John Phillips Memorial Award of the American College of Physicians in April.

Doctor Du Bois' accomplishments and honors are of minor importance compared to the influence he has had upon his students and associates by virtue of his personality and character. As his life-long friend and successor as Professor of Medicine, Dr. David P. Barr, has said, "This extraordinary influence has been attributable only in part to his mastery of experimental procedure and the intrinsic value of his scientific contributions. Its essence derives from his own character and personality. Inspiration has come to others from his abiding faith in principles of scientific and personal conduct, from his integrity and tolerance, and from his sympathetic understanding of the problems of those about him. His character has influenced behavior of his colleagues. It has also influenced innumerable students who have learned from him lessons of critical evaluation, clear expression, unvarying courtesy, and true humility."

Doctor Du Bois was a *gentleman, gentle* in all his dealings with his fellow men and with suffering, and a *man* in his uncompromising attitude toward injustice and dishonesty.

A colleague once asked him why he did not delegate some of his difficult and unpleasant problems to his subordinates. His reply was that such tasks were the duties of the chief.

The principles which guided him in educating the medical students were described in an article entitled "The Clinical Clerkship in Medicine," published in the *Journal of the American Medical Association*, August 21, 1926, and were these: "The purpose of instruction is to teach the students to teach themselves; the manner of instruction is by example and work; the spirit of instruction is sympathy for and faith in the students."

All his friends join his widow, his three children and his nine grandchildren in being proud of their association with a great and good man. His life may be summarized by the citation on the Academy Medal: "Eugene Floyd Du Bois, physiologist, physician, educator, patriot. His life and work have brought honor to the profession of medicine" and to science.

MEMORIAL

JACQUES LOEB

by

W. J. V. Osterhout

This year marks the 100th anniversary of the birth of Jacques Loeb, who contributed so much to the study of marine biology, and it seems appropriate that in a marine biological station where he worked, we should recall his activities.

Thirty-five years have passed since his death and yet our memory of him is still fresh and vivid.

He was above all an idealist. Protected by his devoted wife who knew how to help him, he lived in a world of ideals. Their inspiration dominated his life and set him apart from others. Yet he had also a tender heart, and his sympathy was always with those who were in need of help.

His outstanding feature was his creative imagination, implying prophetic vision, the intuitive, and emotional urge of ideas.

Fortunately his poetic imagination was associated with a keen critical sense. He would test his conceptions over and over again and repeat his experiments very carefully. He published only a small part of his experimental work. It is remarkable that his observations remain valid without fundamental modifications.

The questions he put to nature were never dull and the answers he received were always interesting and at times startlingly so.

He was not content to pursue a special part of a problem without considering its relation to all the rest. To achieve this, it was necessary both to simplify and to generalize, and these powers he possessed to an extraordinary degree.

Courage played a great part in his success. He did not select problems because they were easy but because of their importance. His courage sprang largely from his faith in the mechanistic conception to which he consecrated his life.

He had a truly lovable and sympathetic personality that drew men irresistibly to him. His teaching was inspiring and unforgettable, so that it was not strange that young men gladly followed him.

One felt instinctively that he cared only for truth and that in its quest he would spare no labor and sacrifice.

The breadth of his knowledge made it natural for him to utilize in his work recent advances in other fields of science. Thus he took the ideas of tropism and of heteromorphosis from botany. He applied to biology theories of dissociation and osmotic pressure which resulted in the discovery of artificial parthenogenesis and antagonistic salt action. To the very end of his life he kept in touch with recent progress in physics and chemistry which he applied to his own studies.

Death came while he was actively engaged in what he regarded as the most fundamental investigation of his life. In the midst of this research on proteins he was stricken down.

Here we may pause to ask ourselves, how are we to remember him? He was an idealist, sympathizing with all suffering, consecrating his gifts to humanity; a scientist with an artist's soul, emotional, intuitive, creative; a thinker, strangely original, born to blaze fresh trails and teach new doctrines; a dreamer, regarding the world of life with poetic insight and seeking with creative imagination rarely equalled to sweep aside its mystery and set free the mind of men. His visions, that have made others see visions, cannot but continue to shed inspiration; and in shaping the soul of the future he may serve humanity more than he dared to dream.

MEMORIAL

Frank M. MacNaught

by

Charles Packard

Long and faithful was the service rendered to this Laboratory by Frank M. MacNaught who died in June of this year at the age of 83. Coming to Woods Hole as an accountant in 1913 when the Laboratory began its rapid growth, he was soon made Registrar, and then, in 1916, the Business Manager, a position which he held for 34 years. For much of this time his only assistant was Miss Polly Crowell. Among his many responsibilities was the task of assigning laboratory rooms and tables, and the much more difficult work of apportioning space in the Apartment House and dormitories. Only those who worked closely with him can appreciate the care which he exercised in selecting places best adapted to the needs of each applicant. In addition to these duties, he was responsible for the Mess and its many employees.

From the first he devoted himself to these various tasks, discharging them with great efficiency. Always in the Office, even on Sundays and holidays, he was quick to help newcomers unfamiliar with the operation of the Laboratory. His memory was extraordinary. He could at once call by name investigators and students returning after an absence of many seasons, even recalling the year of their last attendance and the rooms they occupied. His friendliness endeared him to all. He once remarked that if he should take a trip across the country he could spend each night at the home of investigators or students who had especially asked him to visit them. In his relations with all he worked with he showed patience, good judgment, and great tact. Many times he relieved a tense situation with an apt, humorous remark.

He was active in Town affairs, serving on the Finance and other committees, and in the village, as Treasurer and Trustee of the Woods Hole Public Library, and Clerk of the Coonamesset Ranch.

The Laboratory has lost an exceptional man, a devoted friend whose outstanding services and genial personality will long be remembered.

MEMORIAL TO CHARLES R. CRANE ON HIS HUNDREDTH ANNIVERSARY

by

Lawrason Riggs

On the eighth day of August 100 years ago was born the greatest benefactor and friend of the Marine Biological Laboratory, Charles R. Crane.

Mr. Crane was most interested in education. I think this was because he had no formal education beyond grade school. His father, the founder of the Crane Company, did not believe in colleges, in fact he wrote a book against college education, enlisting in the writing of the book two employees of the Crane Company, both of whom turned out to be college graduates.

He went to work at an early age for his father. In 1878 he happened to be in New York. He wandered down to Front Street where in those days the bowsprits of sailing vessels projected over the street. Young Crane went aboard one of these ships and on telegraphic consent from his father arranged to sail on her to Java as the sole passenger. The only additions he made to his baggage were a set of Herbert Spencer and 12 dozen bottles of Guiness Stout. On the voyage he read the Spencer, drank a bottle of stout every day and learned navigation, and on his 21st birthday he furled the main royal in a gale off the Cape of Good Hope. Toward the end of the voyage the first mate died and Mr. Crane was offered his position. He did not accept as he wanted to see as much of Asia as possible. This was fortunate as on the ship's return voyage the captain and most of the crew died, undoubtedly of beri-beri. Later his doctor informed him that his health had been preserved by those 144 bottles of Guiness Stout.

So began his informal education, which he pursued with unrelenting vigor so that in time he became one of the best informed Americans about the Moslem world, Russia and the Far East. During the Peace Conference after World War I he was appointed on a Commission with President King of Oberlin to investigate and make recommendations on the future of Syria. This mission further deepened and extended his contacts with the Near East. Subsequently he was for a number of years American Ambassador to China.

He had an extraordinary interest in exotic places and a real flair for people. He was as much at home in Paris, St. Petersburg, Cairo, Damascus, Constantinople, Samarkand and Pekin as he was in Chicago, and he numbered among his friends, presidents, especially President Wilson, cabinet members, educators, judges, Moslem leaders, including the King of Hejaz and Sherif of Mecca and his son Feisal, later King of Iraq.

He made some 32 visits to Russia, penetrated the most remote parts of Asia, including Bokhara and the Transoxnas, and went with one servant on horseback through Albania after being deserted by the Turkish bodyguard supplied by the Sultan.

The M.B.L. was not the only beneficiary of his interest in education and research. He was interested in the Near East colleges, especially the American College for Girls on the Bosphorus and the Sofia-American Schools in Bulgaria and also a school in Albania and in many universities in this country. The main purpose of his Foundation, The Friendship Fund, was to assist individuals to get an education and the purpose of the Institute of Current World Affairs, which he also founded, was to train specialists in critical areas under conditions that would develop their talents and personality.

His first important contact with the Marine Biological Laboratory was his joining in an offer of assistance with several other persons through President Harper of Chicago University. This happened in 1901.

When this offer and that of the Carnegie Institution were finally rejected by the Laboratory because the trustees and members did not wish to allow the Laboratory to lose its independence, Mr. Crane became more interested through his brother-in-law, Dr. Frank R. Lillie, and before long was contributing about $20,000 a year towards its expenses.

He had a large part in purchasing real property for the Laboratory and in 1913 provided the first brick building, the so-called Crane Building. He had a very important part in the expansion and endowment of the Laboratory between the years 1919 and 1925. He not only capitalized his annual contribution of $20,000 by a gift of $405,000 to endowment, but he guaranteed to pay any cost of the new Rockefeller Building in excess of $500,000. This guarantee cost him $221,000. He had a large part in interesting Mr. John D. Rockefeller, Jr. and the Rockefeller Foundation, as is clear from Mr. Rockefeller's letter found at page 75 of Dr. Lillie's book.

While his gifts were important and always timely, his appreciation of the spirit of the Laboratory and of its democratic and self-governing organization—a group of scientists running their own affairs—was almost more important. He acted as President from 1904 to 1925.

That the Laboratory came through its early and difficult years and survived to become the great institution that it now is is, I feel, largely due to his help and encouragement.

ZOOLOGY

I. CONSULTANTS

F. A. BROWN, JR., Morrison Professor of Zoology, Northwestern University
LIBBIE H. HYMAN, American Museum of Natural History
A. C. REDFIELD, Woods Hole Oceanographic Institution

II. INSTRUCTORS

GROVER C. STEPHENS, Assistant Professor of Zoology, University of Minnesota; in charge of course.

JOHN B. BUCK, Senior Biologist, National Institutes of Health

RALPH I. SMITH, Associate Professor of Zoology, University of California, Berkeley

BERNARD L. STREHLER, Chief, Cellular and Comparative Physiology, Division of Gerontology, National Institutes of Health

PAUL P. WEINSTEIN, Laboratory of Tropical Diseases, National Institutes of Health

RICHARD C. SANBORN, Professor of Zoology, Department of Biological Sciences, Purdue University

Morris Rockstein, Associate Professor of Physiology, New York University College of Medicine
Milton Fingerman, Assistant Professor of Zoology, Tulane University

III. Laboratory Assistants

Robert Ashman, Wabash College
Donald Hall, University of Michigan

EMBRYOLOGY

I. Instructors

Mac V. Edds, Jr., Professor of Biology, Brown University; in charge of course
Philip Grant, Assistant Professor of Pathobiology, Johns Hopkins University
John W. Saunders, Jr., Professor of Zoology, Marquette University
Nelson T. Spratt, Jr., Professor of Zoology, University of Minnesota
Maurice Sussman, Associate Professor of Biological Sciences, Brandeis University
Lionel Rebhun, Assistant Professor of Biology, Princeton University

II. Laboratory Assistants

Chandler M. Fulton, Rockefeller Institute for Medical Research
David S. Love, University of Colorado

PHYSIOLOGY

I. Consultants

Merkel H. Jacobs, Professor of Physiology, University of Pennsylvania
Arthur K. Parpart, Professor of Biology, Princeton University
Albert Szent-Györgyi, Director, Institute for Muscle Research, Marine Biological Laboratory

II. Instructors

W. D. McElroy, Professor of Biology, Johns Hopkins University; in charge of course
Francis D. Carlson, Associate Professor of Biophysics, Johns Hopkins University
Bernard D. Davis, Professor of Bacteriology, Harvard Medical School
Donald Griffin, Professor of Zoology, Harvard University
Howard Schachman, Virus Laboratory, University of California
Timothy Goldsmith, Fellow, Harvard University
Robert Loftfield, Massachusetts General Hospital

III. Laboratory Assistant

Louis Otero, University of Puerto Rico, Rio Piedras

BOTANY

I. Consultant

Wm. Randolph Taylor, Professor of Botany, University of Michigan

II. Instructors

Richard C. Starr, Associate Professor of Botany, Indiana University; in charge of course
John M. Kingsbury, Assistant Professor of Botany, Cornell University
Walter R. Herndon, Assistant Professor of Biology, University of Alabama

III. Collector

G. Benjamin Bouck, Columbia University

IV. Laboratory Assistants

Larry Hoffman, University of Texas
Robert W. Korn, Indiana University

ECOLOGY

I. Consultants

Paul Galtsoff, U. S. Fish and Wildlife Service, Woods Hole
Alfred C. Redfield, Woods Hole Oceanographic Institution
Bostwick H. Ketchum, Woods Hole Oceanographic Institution
Edwin T. Moul, Rutgers University
Charles E. Jenner, University of North Carolina
Howard L. Sanders, Woods Hole Oceanographic Institution

II. Instructors

Eugene P. Odum, Alumni Foundation Professor of Zoology, University of Georgia; in charge of course
Howard T. Odum, University of Texas
Harold J. Humm, Associate Professor of Botany, Duke University
John H. Ryther, Marine Biologist, Woods Hole Oceanographic Institution

III. Laboratory Assistant

Richard B. Williams, Harvard University

1. The Laboratory Staff, 1959

Homer P. Smith, General Manager

Mrs. Deborah Lawrence Harlow, Librarian
Carl O. Schweidenback, Manager of the Supply Department
Irvine L. Broadbent, Office Manager
Robert Kahler, Superintendent, Buildings and Grounds
Robert B. Mills, Manager, Department of Research Service

GENERAL OFFICE

Mrs. Lila S. Myers
Mrs. Vivien R. Brown
Mrs. Virginia M. Morehouse
Mrs. Loretta J. McCartney
Mrs. Marion C. Chase
Mrs. Vivian I. Manson
Mrs. Shirley A. Elder

LIBRARY

Mrs. M. Verna Hanks
Mrs. Gwendolyn S. Blomberg
Mrs. Naomi Botelho
Albert K. Neal

MAINTENANCE OF BUILDINGS AND GROUNDS

Robert Adams
Eldon P. Allen
Arthur D. Callahan
Robert Gunning
Walter J. Jaskun
Donald B. Lehy
Ralph H. Lewis
Russell F. Lewis
Alan G. Lunn
Alton J. Pierce
Robert H. Walker, Jr.
James S. Thayer

DEPARTMENT OF RESEARCH SERVICE

Gail M. Cavanaugh
John P. Harlow
Seaver R. Harlow
Mrs. Arlene Brown

SUPPLY DEPARTMENT

Donald P. Burnham
Milton B. Gray
Geoffrey J. Lehy
Robert O. Lehy
Mrs. Mildred H. Mixson
Robert M. Perry
Bruno F. Trapasso
John J. Valois
Jared L. Vincent
Hallett S. Wagstaff

2. Investigators, Lalor and Lillie Fellows, and Students

Independent Investigators, 1959

Adelman, William J., Assistant Professor of Physiology, University of Buffalo
Allen, Robert D., Assistant Professor of Biology, Princeton University
Amberson, William R., Investigator, Marine Biological Laboratory
Armstrong, Philip B., Professor and Chairman of Anatomy, State University of New York, College of Medicine at Syracuse
Arnold, William, Principal Biologist, Oak Ridge National Laboratory
Baltus, Elyane, Chargé de Course, University of Brussels, Belgium
Bang, Frederik B., Professor of Pathobiology, Johns Hopkins University School of Hygiene
Barth, L. G., Professor of Zoology, Columbia University
Baylor, Martha B., Independent Investigator, Marine Biological Laboratory
Bell, Eugene, Assistant Professor of Biology, Massachusetts Institute of Technology
Benesch, Reinhold, Investigator, Marine Biological Laboratory
Benigna, Sister Maria, Professor of Biology, Saint Joseph College
Bennett, Michael, Research Associate, Columbia University, College of Physicians and Surgeons
Bennett, Miriam F., Assistant Professor of Biology, Sweet Briar College
Benzer, Seymour, Professor of Biophysics, Purdue University
Bergmann, Felix, Research Fellow, College of Physicians and Surgeons, Columbia University
Berman, Mones, Physicist, National Institutes of Health
Bernard, George R., Assistant Professor of Biology, University of Notre Dame
Bernstein, Maurice H., Assistant Professor of Anatomy, Wayne State University
Bishop, David W., Staff Member, Carnegie Institution of Washington
Bosler, Robert, Instructor of Physiological Optics, Johns Hopkins Hospital
Brett, William J., Associate Professor of Biology, Indiana State Teachers College
Brown, Frank A., Jr., Morrison Professor of Biology, Northwestern University

Bryant, Shirley H., Assistant Professor of Pharmacology, University of Cincinnati
Buck, John B., Physiologist, National Institutes of Health, Laboratory of Physical Biology
Burbanck, W. D., Professor of Biology, Emory University
Burk, Rev. Joseph A., Assistant Professor, Loyola College
Cabrera, Guillermo, Assistant Professor of Biochemistry, New York University College of Medicine
Carlson, Francis D., Associate Professor of Biophysics, Johns Hopkins University
Cascarano, Joseph, Instructor of Pathology, New York University College of Medicine
Case, James, Assistant Professor of Zoology, State University of Iowa
Chaet, Alfred B., Associate Professor of Biology, American University
Cheney, Ralph Holt, Professor of Biology, Brooklyn College
Child, Frank M., Instructor in Zoology, University of Chicago
Claff, C. Lloyd, Research Associate in Surgery, Harvard Medical School
Cole, Kenneth S., Chief, Laboratory of Biophysics, National Institutes of Health
Colwin, Arthur L., Professor of Biology, Queens College
Colwin, Laura H., Lecturer, Queens College
Cooperstein, Sherwin J., Associate Professor of Anatomy, Western Reserve University School of Medicine
Costello, Donald P., Kenan Professor of Zoology, University of North Carolina
Crane, Robert K., Associate Professor of Biological Chemistry, Washington University Medical School
Crosti, Nicoletta, Foreign Italian Student, Bryn Mawr College
Crowell, Sears, Associate Professor of Zoology, Indiana University
Csapo, Arpad, Associate Professor, Rockefeller Institute for Medical Research
Davis, Bernard, Professor of Bacteriology, Harvard Medical School
Doolin, Paul F., Assistant Professor of Biology, Washington and Jefferson College
DuBois, Arthur B., Associate Professor of Physiology, University of Pennsylvania School of Medicine
Echalier, Guy P. R., Research Fellow in Biology, Harvard College
Edds, Mac V., Jr., Professor of Biology, Brown University
Edmonds, Mary, Research Associate, Montefiore Hospital Research Institute
Failla, G., Professor of Radiology, Columbia University
Feigelson, Philip, Assistant Professor of Biochemistry, College of Physicians and Surgeons
Fingerman, Milton, Assistant Professor of Zoology, Newcomb College of Tulane University
Fishman, Louis, Research Associate, New York University College of Dentistry
Forrest, Hugh S., Associate Professor of Zoology, University of Texas
Frank, Ulrich, Extraordinary Professor, Technical University, Darmstadt, Germany
Friz, Carl T., Public Health Fellow, University of Minnesota
Fujimori, Eiji, Investigator, Institute for Muscle Research, Marine Biological Laboratory
Fuortes, M. G. F., Physiologist, National Institutes of Health
Furshpan, Edwin, Instructor in Ophthalmic Physiology, Johns Hopkins University
Furukawa, Taro, Instructor in Ophthalmic Physiology, Johns Hopkins University
Galtsoff, Paul S., Director, Shellfish Laboratory, U. S. Bureau of Commercial Fisheries
Gardella, Joseph W., Assistant Dean, Harvard Medical School
Glade, Richard W., Assistant Professor of Zoology, University of Vermont
Goldsmith, Timothy H., Junior Fellow, Harvard University
Gonse, Pierre H., Research Fellow, University of Pennsylvania
Goreau, Thomas F., Lecturer in Physiology, University College of the West Indies
Gorini, Luigi, Lecturer, Harvard Medical School
Grant, Philip, Assistant Professor of Pathobiology, Johns Hopkins University School of Hygiene
Gray, I. E., Professor of Zoology, Duke University
Green, James W., Associate Professor of Physiology, Rutgers, The State University
Greif, Roger L., Associate Professor of Physiology, Cornell University Medical College
Griffin, Donald R., Professor of Zoology, Harvard University
Grosch, Daniel S., Professor of Genetics, North Carolina State College
Gross, Paul Randolph, Associate Professor of Biology, New York University
Gross, Samson Richard, Assistant Professor, Rockefeller Institute for Medical Research

Grundfest, Harry, Associate Professor of Neurology, College of Physicians and Surgeons, Columbia University
Guttman, Rita, Associate Professor of Biology, Brooklyn College
Harvey, Ethel Browne, Investigator in Biology, Princeton University
Harvey, E. Newton, Professor of Physiology, Emeritus, Princeton University
Hay, Elizabeth D., Assistant Professor of Anatomy, Cornell University Medical College
Hayashi, Teru, Professor of Zoology, Columbia University
Hegyeli, Andrew, Investigator, Institute for Muscle Research, Marine Biological Laboratory
Heilbrunn, L. V., Professor of Zoology, University of Pennsylvania
Henley, Catherine, Research Associate, University of North Carolina
Herndon, Walter R., Assistant Professor of Biology, University of Alabama
Hervey, John P., Senior Electronic Engineer, Rockefeller Institute
Hiatt, Howard H., Assistant Professor of Medicine, Harvard Medical School
Hibbard, Hope, Professor, Oberlin College
Hill, Robert B., Instructor in Zoology, University of Maine
Holz, George G., Jr., Associate Professor of Zoology, Syracuse University
Humm, Harold J., Associate Professor of Botany, Duke University
Hursh, John B., Professor of Radiation Biology, University of Rochester
Hurwitz, Jerard, Assistant Professor of Microbiology, New York University
Isenberg, Irvin, Investigator, Institute for Muscle Research, Marine Biological Laboratory
Jacobs, William P., Associate Professor of Biology, Princeton University
Jenner, Charles E., Associate Professor and Chairman of Zoology, University of North Carolina
Jones, Mary Ellen, Assistant Professor of Biochemistry, Brandeis University
Jones, Raymond F., Visiting Research Associate, Marine Biological Laboratory
Kaminer, Benjamin, Senior Lecturer in Physiology, University of Witwatersrand, Johannesburg, South Africa
Kane, Robert E., Assistant Professor of Biochemistry, Brandeis University
Kaplan, Nathan O., Professor and Chairman of Biochemistry, Brandeis University
Kempton, Rudolf T., Chairman, Department of Zoology, Vassar College
Kingsbury, John M., Assistant Professor of Botany, Cornell University
Kleinholz, L. H., Professor of Biology, Reed College
Köhler, Kurt, Research Associate, Florida State University
Kuffler, Stephen W., Investigator, Johns Hopkins Hospital
Kuriyama, Hirosi, Rockefeller Institute for Medical Research
Kury, Livia Rev, Investigator, Institute for Muscle Research, Marine Biological Laboratory
Lansing, Albert I., Professor and Chairman of Anatomy, University of Pittsburgh
Lash, James W., Associate in Anatomy, University of Pennsylvania, School of Medicine
Lazarow, Arnold, Professor and Head, Dept. of Anatomy, University of Minnesota
Leone, Vincenzo, Professor, Istituto di Zoologia, Milano, Italy
Levy, Milton, Professor and Chairman, Dept. of Biochemistry, New York University College of Dentistry
Lewin, Ralph Arnold, Investigator, Marine Biological Laboratory
Lochhead, John H., Professor of Zoology, University of Vermont
Loftfield, Robert B., Associate in Organic Chemistry, Harvard Medical School
Lorand, L., Associate Professor of Chemistry, Northwestern University
Love, Warner E., Assistant Professor of Biophysics, Johns Hopkins University
Lowenhaupt, Benjamin, Research Associate, Rockefeller Institute for Medical Research
McElroy, William D., Head, McCollum-Pratt Institute, Johns Hopkins University
Marks, Paul A., Assistant Professor of Medicine, College of Physicians and Surgeons, Columbia University
Marsh, Julian B., Assistant Professor of Biochemistry, University of Pennsylvania
Marshall, Jean M., Assistant Professor of Physiology, Johns Hopkins University School of Medicine
Marsland, Douglas, Professor of Biology, New York University, Washington Square College
Mateyko, Gladys M., Assistant Professor of Biology, New York University, Washington Square College
Metz, Charles B., Professor, Florida State University

Middlebrook, W. Robert, Research Fellow, Institute for Muscle Research, Marine Biological Laboratory
Monier, Roger, Post-doctoral Associate, University of Paris
Moore, John W., Associate Chief, Laboratory of Biophysics, National Institutes of Health
Nace, Paul Foley, Associate Professor of Biology, McMaster University
Nelson, Leonard, Assistant Professor, University of Chicago
Newton, Jack W., Research Associate, Brandeis University
Odum, Eugene P., Professor of Zoology, University of Georgia
Osterhout, W. J. V., Member *Emeritus,* Rockefeller Institute for Medical Research
Palincsar, Edward E., Instructor of Biology, Loyola University
Papaconstantinou, John, Post-doctoral Research Fellow, Carnegie Institution of Washington
Parpart, Arthur K., Chairman, Department of Biology, Princeton University
Paterson, Mabel C., Assistant Professor of Zoology, Vassar College
Perlmann, Gertrude E., Associate Professor, Rockefeller Institute for Medical Research
Prosser, C. Ladd, Professor of Physiology, University of Illinois
Ranzi, Silvio, Full Professor, Istituto di Zoologia, Milano, Italy
Rapport, Maurice M., Professor of Biochemistry, Albert Einstein College of Medicine
Rasmussen, Howard, Graduate Fellow, Rockefeller Institute for Medical Research
Read, Clark P., Associate Professor of Parasitology, Johns Hopkins University
Rebhun, Lionel I., Assistant Professor of Biology, Princeton University
Rieser, Peter, Research Associate, University of Pennsylvania
Rockstein, Morris, Associate Professor of Physiology, New York University College of Medicine
Rose, S. Meryl, Professor of Zoology, University of Illinois
Rosenberg, Evelyn E., Associate Professor of Pathology, New York University-Bellevue Medical Center
Roslansky, John D., Research Associate, Princeton University
Roth, Jay S., Associate Professor of Biochemistry, Hahnemann Medical College
Rothman, Alvin H., Research Fellow, Johns Hopkins University School of Hygiene
Rudomin, P., Research Fellow, College of Physicians and Surgeons, Columbia University
Rushton, W. A. H., Reader in Physiology, Trinity College, Cambridge, England
Rustad, Ronald C., Instructor in Physiology, Florida State University
Ryther, John H., Staff, Woods Hole Oceanographic Institution
Sanborn, Richard C., Professor of Zoology, Purdue University
Sandeen, Muriel I., Assistant Professor of Zoology, Duke University
Sanders, Howard L., Research Associate, Woods Hole Oceanographic Institution
Saunders, John W., Professor of Zoology, Chairman Department of Biology, Marquette University
Schachman, Howard K., Associate Professor of Biochemistry, University of California, Berkeley
Schuh, Rev. Joseph E., Associate Professor of Biology and Chairman of Department, Saint Peter's College
Scott, Sister Florence Marie, Professor of Biology, Seton Hill College
Scott, George T., Professor and Chairman, Department of Biology, Oberlin College
Seliger, Howard H., Guggenheim Fellow, Johns Hopkins University
Skoglund, Carl Rudolf, Associate Professor, Karolinska Institutet, Stockholm, Sweden
Smith, Paul Ferris, Electronics Engineer, Rockefeller Institute for Medical Research
Smith, Ralph I., Associate Professor of Zoology, University of California, Berkeley
Spector, Abraham, Instructor, Harvard Medical School
Speidel, Carl C., Professor and Chairman of Anatomy, University of Virginia
Spiegel, Melvin, Assistant Professor of Biology, Colby College
Spratt, Nelson T., Chairman, Department of Zoology, University of Minnesota
Spyropoulos, Constantine S., Neurophysiologist, National Institutes of Health
Starr, Richard C., Associate Professor of Botany, Indiana University
Steele, Richard, Associate Professor of Biochemistry, Tulane University
Steinbach, H. Burr, Professor and Chairman, Department of Zoology, University of Chicago
Steinhardt, Jacinto, Director, Operations Evaluation Group, Massachusetts Institute of Technology

Stephens, Grover C., Associate Professor of Zoology, University of Minnesota
Stetten, DeWitt, Associate Director in Charge of Research, National Institutes of Health
Stetten, Marjorie R., Biochemist, National Institutes of Health
Stevens, Charles F., Medical Student, Yale University School of Medicine
Stone, William, Director of Ophthalmic Plastics Laboratory, Massachusetts Eye and Ear Infirmary
Strehler, Bernard L., Chief, Comparative Physiology Section, National Institutes of Health
Strittmatter, Philipp, Assistant Professor of Biochemistry, Washington University
Stunkard, Horace W., Research Scientist, U. S. Fish and Wildlife Service
Sudak, Frederick N., Instructor in Physiology, Albert Einstein College of Medicine
Sussman, Maurice, Associate Professor, Brandeis University
Szent-Györgyi, Albert, Director, Institute for Muscle Research, Marine Biological Laboratory
Szent-Györgyi, Andrew, Investigator, Institute for Muscle Research, Marine Biological Laboratory
Tasaki, Ichiji, Chief, Special Senses Section, National Institutes of Health
Taylor, Robert E., Neurophysiologist, National Institutes of Health
Taylor, Wm. Randolph, Professor of Botany, University of Michigan
Teorell, Torsten, Professor of Physiology, Uppsala University, Sweden
Torch, Reuben, Assistant Professor of Zoology, University of Vermont
Troll, Walter, Assistant Professor of Industrial Medicine, New York University-Bellevue Medical Center
Tsuboi, Kenneth K., Assistant Professor of Biochemistry, Cornell University Medical College
Tweedell, Kenyon S., Assistant Professor of Biology, University of Notre Dame
deVillafranca, George W., Assistant Professor of Zoology, Smith College
Villee, Claude A., Associate Professor of Biological Chemistry, Harvard University
Vincent, Walter S., Assistant Professor of Anatomy, Upstate Medical Center, State University of New York
Wainio, Walter W., Associate Professor of Biochemistry, Rutgers University
Watanabe, Akira, Research Fellow, College of Physicians and Surgeons, Columbia University
Webb, H. Marguerite, Assistant Professor of Biology, Goucher College
Weinstein, Paul P., Senior Scientist, National Institutes of Health
Weiss, Leon P., Assistant Professor of Anatomy, Harvard Medical School
Wells, G. P., Professor of Zoology, University College, London, England
Werman, Robert, Research Associate, College of Physicians and Surgeons, Columbia University
Whiting, Anna R., Guest Investigator, University of Pennsylvania
Wichterman, Ralph, Professor of Biology, Temple University
Wiercinski, Floyd J., Research Associate, University of Pennsylvania
Willey, C. H., Professor of Biology and Chairman of Department, New York University
Wilson, Walter L., Assistant Professor of Physiology, University of Vermont College of Medicine
Wittenberg, Jonathan B., Assistant Professor of Physiology, Albert Einstein College of Medicine
Wright, Paul A., Associate Professor of Zoology, University of New Hampshire
Zweifach, B. W., Professor of Pathology, New York University-Bellevue Medical Center
Zwilling, Edgar, Associate Professor, University of Connecticut

Lalor Fellows, 1959

Bernstein, M. H., Wayne State University
Crosti, Nicoletta, Bryn Mawr College
Echalier, G. P. R., Harvard College
Gonse, P. H., University of Pennsylvania
Leone, V., Istituto di Zoologia, Milano, Italy
Marsh, J. B., University of Pennsylvania
Nelson, Leonard, University of Chicago
Palincsar, E. E., Loyola University
Ranzi, Silvio, Istituto di Zoologia, Milano, Italy
Roslansky, J. D., Princeton University

Rustad, R. C., Florida State University
Skoglund, Carl, Karolinska Institutet, Stockholm, Sweden
Strittmatter, P., Washington University

Lillie Fellow

Ranzi, Silvio, Istituto di Zoologia, Milano, Italy

Grass Fellows

Lipicky, Raymond, University of Cincinnati
Stevens, Charles, Yale University, School of Medicine

Beginning Investigators, 1959

Bensam, Bertrand J., State University of New York, Upstate Medical Center, Syracuse
Broberg, Patricia L., Brandeis University
Burnstock, G., University of Illinois
Butterworth, Frank M., University of Pennsylvania
Byers, Thomas J., University of Pennsylvania
Campbell, James Wayne, Johns Hopkins University
Carlson, Albert D., State University of Iowa
Chernetski, Kent Eugene, University of California
Curtis, Brian A., Rockefeller Institute for Medical Research
Davidson, Morton, New York University Medical College
Dubnau, David, Columbia University
Dudel, Josef, Johns Hopkins University
Faust, Robert Gilbert, Princeton University
Filosa, Michael, Princeton University
Graham, Charles Edward, Johns Hopkins University School of Medicine
Griffin, Dean H., American University
Gump, Dieter W., Johns Hopkins University
Guttman, Burton S., Institute of Molecular Biology, University of Oregon
Hurwitz, Charles, VA Hospital, Albany
Huver, Charles W., Yale University
Jackson, James A., Western Reserve University
Kuperman, Albert S., Cornell University Medical College
Laurie, John S., Tulane University
Lipicky, Raymond John, University of Cincinnati
de Lorenzo, A. J., Johns Hopkins University Medical School
Nagler, Arnold L., New York University-Bellevue Medical Center
Narbaitz, Roberto, Carnegie Institution of Washington and Universidad de Buenos Aires
Orkand, Richard K., University of Utah
Pepe, Frank A., University of Pennsylvania
Polgar, George, University of Pennsylvania School of Medicine
Potter, David, Johns Hopkins University
Reuben, John P., College of Physicians and Surgeons, Columbia University
Rubin, Arnold D., New York University College of Medicine
Schafer, David G., New York University College of Medicine
Shephard, David, University of Chicago
Smith, Thomas G., Jr., College of Physicians and Surgeons, Columbia University
Smyth, Thomas, Jr., Pennsylvania State University
Sudduth, Solon Scott, Johns Hopkins University School of Medicine
Swami, Karumuri S., University of Pennsylvania
Theorell, Klas T. G., Karolinska Institutet, Stockholm, Sweden
Tobin, Michael, New York University Medical Center, Downstate
Warner, Eldon D., University of Wisconsin

Wertheim, Guta, Hebrew University
Wheeler, James English, Johns Hopkins University School of Medicine
Winick, Paul, Columbia University

Research Assistants, 1959

Allen, Constance, Massachusetts Eye and Ear Infirmary
Ashman, Robert F., Wabash College
Ashton, Francis T., University of Pennsylvania
Asterita, Harvey L., New York University
Baird, Spencer, Marine Biological Laboratory
Barnwell, Franklin H., Northwestern University
Barron, Evelyn, Massachusetts Eye and Ear Infirmary
Berman, Lawrence J., Princeton University
Blumstein, Joyce R., Albert Einstein College of Medicine
Boleyn, Brenda J., University of Rhode Island
Bouck, G. Benjamin, Columbia University
Branham, Joseph, Florida State University
Braverman, Maxwell H., University of Illinois
Brooks, Kenneth H., Indiana University
Bunim, Lesley S., Barnard College
Cicak, Anna, Albert Einstein College of Medicine
Clark, Eloise E., University of California
Clark, Lynne G., Queens College
Conway, Dorothy M., Rockefeller Institute for Medical Research
Corlette, Sally L., Institute for Cancer Research
Cousineau, Gilles, University of New York
Delson, Rozanne, Massachusetts Institute of Technology
Dingle, Al D., University of Illinois
Doolittle, Russell F., Harvard University
Doss, Dicky E., American University
Dunsky, Milton H., Rockefeller Institute for Medical Research
Eigner, Elizabeth Ann, Massachusetts General Hospital
Ein, Daniel, New York University-Bellevue Medical Center
Erskine, Louise, Institute for Muscle Research, Marine Biological Laboratory
Esper, Hildegard, Columbia University
Feldherr, Carl M., University of Pennsylvania
Fielden, Ann, University of Illinois
Finkel, Arnold, New York University College of Medicine
Fiorentino, Eileen, Hahnemann Medical College
Fisher, Frank M., Jr., Purdue University
Foran, Elizabeth H., Smith College
Friedler, Gladys, Tufts Medical School
Friedman, Leonard, Rutgers University
Fulton, Chandler M., Rockefeller Institute for Medical Research
Gasseling, Mary T., Marquette University
Gebhart, John H., National Institutes of Health
Goldfarb, David, Johns Hopkins University
Goudsmit, Esther M., University of Michigan
Griffin, Joe L., Princeton University
Haas, Florence Anne, Western University Medical School
Haley, Barbara, Brandeis University
Hall, Donald J., University of Michigan
Hampson, George Richard, Northeastern University
Hanson, Frank E., Jr., State University of Iowa
Haskell, Judith Ann, Purdue University
Hathaway, Ralph R., Florida State University

Hike, Sally Jayne, Mount Holyoke College
Hillman, Celia A., Harvard University
Himmelfarb, Sylvia, University of Maryland School of Medicine
Hoffman, Larry R., University of Texas
Holsten, George H., III, Rutgers University
Holt, Charles E., III, Massachusetts Institute of Technology
Jackson, Thomas John, Lehigh University
Johnson, Christine A., Wheaton College
Kaighn, Morris E., Massachusetts Institute of Technology
Korn, Robert William, Indiana University
Lamont, Hayes C., Columbia University
Leighton, Charles, Colby College
Libbin, Dick, Bard College
Lippert, Byron E., Indiana University
Lonigro, Norma, Seton Hill College
Loring, Janet, Harvard Medical School
Love, David S., University of Colorado
McConnaughy, R. A., American University
McGowan, Bernard L., Johns Hopkins University
McLaughlin, Jane, Institute for Muscle Research, Marine Biological Laboratory
Mangum, Charlotte Preston, Vassar College
Makinen, Paula, University of Minnesota
Malkoff, Donald B., University of Pittsburgh Medical School
Mavridis, Paraskevi J., Purdue University
Merrill, Charlotte F., Massachusetts Institute of Technology
Merson, Gerald, New York University
Mingioli, Elizabeth S., Harvard University
Mobberly, William C., Tulane University
Morrison, Roberta Anne, Smith College
Moule, John William, McMaster University
Moule, Margaret, McMaster University
Mueller, Helmut, Institute for Muscle Research, Marine Biological Laboratory
Otero-Vilardebo, Luis, University of Puerto Rico
Palubinkas, Bertha, College of Physicians and Surgeons, Columbia University
Perry, Barbara, Institute for Muscle Research, Marine Biological Laboratory
Philpott, Charles W., Tulane University
Reich, Melvin, Rutgers University
Reuben, John Phillip, University of Florida
Roberts, Mary Lou, Washington University Medical School
Rogers, Annette, North Carolina State College
Rose, Jeannette, Bates College
Rosenbluth, Raja, Columbia University
Rothstein, Howard, University of Pennsylvania
Saturen, Janice, State University of New York Upstate Medical Center at Syracuse
Schroeder, Paul C., St. Peter's College
Schuel, Herbert, University of Pennsylvania
Sellers, Richard Lee, American University
Siegel, Paula, University of Cincinnati
Siger, Alvin, Johns Hopkins University
Silkovskis, Izolde, McMaster University
Simmons, John E., Johns Hopkins University
Spencer, Joyce M., Harvard Medical School
Staub, Herbert W., Rutgers University
Stoll, Louise, Johns Hopkins University School of Hygiene
Swope, Julia, Massachusetts General Hospital
Szent-Györgyi, Eva, Institute for Muscle Research, Marine Biological Laboratory
Szent-Györgyi, Marta, Institute for Muscle Research, Marine Biological Laboratory

Teyan, Fred, Albert Einstein College of Medicine
Thomas, Cynthia, Massachusetts Eye and Ear Infirmary
VanLiew, Hugh D., U. S. Navy, Bethesda
vanNorman, Earl, Princeton University
Wahbe, Vera, Kansas University
Watkins, Dudley T., Oberlin College
Yates, Lloyd Austin, University of Minnesota
Yip, Cecil C., McMaster University

Library Readers 1959

Ball, Eric G., Professor of Biological Chemistry, Harvard Medical School
Baylor, Martha B., Investigator, Marine Biological Laboratory
Beidler, Lloyd M., Professor of Physiology, Florida State University
Bodansky, Oscar, Chief, Division of Metabolism and Enzyme Studies, Sloan-Kettering Institute
Brown, Dugald, Professor of Zoology, University of Michigan
Butler, Elmer G., Professor of Biology, Princeton University
Chase, Aurin M., Associate Professor of Biology, Princeton University
Clark, Eliot R., University of Pennsylvania
Cohen, Seymour S., Professor of Biochemistry, University of Pennsylvania School of Medicine
Collier, Jack R., Marine Biological Laboratory
Foerster, Theodor, Professor of Physical Chemistry, Technische Hochschule, Stuttgart, W. Germany
Fries, E. F. B., Associate Professor, City College of New York
Gabriel, Mordecai L., Associate Professor of Biology, Brooklyn College
Gaffron, Hans, Professor of Biochemistry, University of Chicago
Ginsberg, Harold S., Associate Professor of Preventive Medicine, Western Reserve University
Goldthwait, David A., Assistant Professor of Biochemistry, Western Reserve University
Hunter, F. R., Professor and Head, Dept. of Biology, Univ. de los Andes, Bogota, Colombia
Jacobs, M. H., Professor Emeritus, University of Pennsylvania
Karush, Fred, Professor of Immunochemistry, University of Pennsylvania
Kasha, Michael, Professor of Chemistry, State University of Florida
Klein, Morton, Professor of Microbiology, Temple University School of Medicine
Kozloff, Lloyd M., Associate Professor of Biochemistry, University of Chicago
Leighton, Joseph, Associate Professor of Pathology, University of Pittsburgh School of Medicine
Lubin, Martin, Assistant Professor of Pharmacology, Harvard Medical School
Ludwig, George D., Assistant Professor of Medicine, University of Pennsylvania
McDonald, Sister Elizabeth Seton, Professor of Biology, College of Mt. St. Joseph on the Ohio
Minard, Frederick, Research Biochemist, Abbott Laboratories
Moul, Edwin T., Associate Professor of Botany, Rutgers University
Musacchia, X. J., Associate Professor in Biology, St. Louis University
Novikoff, Alex B., Research Professor, Albert Einstein College of Medicine
Pullman, Bernard, Professor of Theoretical Chemistry, University of Paris, France
Rhuland, Lionel E., Research Section Head, The Upjohn Company
Rochovansky, Olga M., Research Assistant, Public Health Research Institute of New York City
Root, Walter S., Professor of Physiology, College of Physicians and Surgeons, Columbia University
Roth, Fr. Owen H., Associate Professor of Zoology, St. Vincent College
Schlamowitz, Max, Associate Cancer Research Scientist, Roswell Park Memorial Institute
Serber, Barbara Jo, Assistant Professor of Anatomy, New York University-Bellevue Medical Center
Sonnenblick, B. P., Professor of Biology, Rutgers University
Sulkin, S. Edward, Professor and Chairman, Dept. of Microbiology, University of Texas, Southwestern Medical School

Trurnit, Hans J., Senior Scientist, Research Institute for Advanced Study
Warner, Robert C., Associate Professor of Biochemistry, New York University College of Medicine
Weigle, William O., Assistant Research Professor, University of Pittsburgh School of Medicine
Wheeler, George E., Instructor in Biology, Brooklyn College
Yntema, Chester L., Professor of Anatomy, State University of New York, Upstate Medical Center
Zinn, Donald J., Associate Professor of Zoology, University of Rhode Island

Students 1959

BOTANY

Brown, Malcolm, University of Texas
Churchill, Algernon C., Harvard University
Correll, David L., Michigan State University
Edwards, Jackie L., University of Alabama
Ehrlich, Diana Lee, College of the City of New York
Findley, Davis L., University of Alabama
Flach, Mary E., Vassar College
Foldats, Ernesto, Universidad Central de Venezuela
Fredericks, Walter W., Johns Hopkins University
Golas, Mary, Marquette University
Kalil, Mildred, Wellesley College
Koob, Derry Delos, Cornell University
Mason, Charles P., Cornell University
Miles, Marjorie L., Acadia University
Morris, Ruth Carol, Cornell University
Nolan, Richard A., University of Nebraska
Shor, Bernice C., Rollins College
Wagner, Kenneth A., College of William and Mary
Williams, Richard B., Harvard University
Zacharia, Kuruvila, Princeton University

EMBRYOLOGY

Ashman, Robert F., Wabash College
Baker, John R., University of Minnesota
Bergmann, Fred H., Brandeis University
Birky, C. William, Jr., Indiana University
Cordes, Eugene H., Brandeis University
Curtis, Joseph C., Brown University
Gibley, Charles W., Jr., Iowa State College
Grand, Theodore I., Brown University
Grinnell, Alan D., Harvard University
Harris, Thomas M., University of North Carolina
Hennen, Sally H., Indiana University
Holt, Charles E., III, Massachusetts Institute of Technology
Kessler, Dietrich, University of Wisconsin
Lawrence, Irvin E., Kansas University
Lessups, Roland J., S. J., Johns Hopkins University
Levine, Stephen, Brandeis University
Merson, Gerald, New York University Medical School
Pierce, Gordon B., University of Pittsburgh
Rose, Irwin A., Yale University
Schuler, Margery E., Wesleyan University

Steinberg, Sonia Naomi, Northwestern University
Whittaker, J. Richard, Yale University
Yates, Robert D., University of Alabama Medical Center

PHYSIOLOGY

Alvaredo, Francisco, New York University, College of Medicine
Angeles, Leticia, Tulane University
Benjamin, Thomas, Amherst College
Eisen, James, Emory University
Garrick, Michael, Johns Hopkins University
Gillespie, Barbara, Radcliffe University
Gottlieb, Abraham, New York University-Bellevue Medical Center
Green, Morris, University of Rochester
Hamilton, Mary, Sloan-Kettering Institute
Handler, Joseph, University of Pennsylvania
Homer, Louis, Medical College of Virginia
Kaley, Gabor, New York University
Kean, Edward, University of Pennsylvania
Kinsolving, Clyde, Vanderbilt University
Liebman, Paul, Barnes Hospital
Luchi, Robert, University of Pennsylvania
Plotz, Paul, Harvard Medical School
Purple, Richard, Rockefeller Institute
Rosenbaum, Joel, Syracuse University
Ryser, Hugues, Massachusetts General Hospital
Slayman, Clifford, Rockefeller Institute
Theorell, Henning, Karolinska Inst., Stockholm
Todaro, George, New York University College of Medicine
Townsend, Edith, McGill University
Walch, Carolyn, Johns Hopkins University
Weiss, Charles, Harvard University
Whittenbury, Guillermo, Harvard Medical School

INVERTEBRATE ZOOLOGY

Andrew, Oliver T., Franklin and Marshall College
Berchmans, Sister Ann, St. Mary of the Woods College
Brebbia, Dante R., Fordham University Graduate School
Brenowitz, Harry, Adelphi College
Buckley, Brother William, Fordham University
Churchill, Algernon, Harvard University
Conrow, Mary M., Wilson College
Correll, David, Michigan State University
DeLong, Karl T., Oberlin College
Eddy, Jane, Tufts University
Edward, Brother C., Fordham University
Ellison, Esther, University of Minnesota
Englund, Paul, Hamilton College
Epel, David, University of California, Berkeley
Feir, Dorothy J., University of Wisconsin
Ferguson, John, Cornell University
Gage, Elizabeth M., Cushing Academy
Gates, David A., Clark University
Goldman, Lawrence, University of California, Los Angeles
Greene, Laurel E., Goucher College
Gutknecht, John, University of North Carolina

Hayes, William, University of Michigan
Henderson, Oliver, Jr., The Citadel
Huber, Sally A., Mt. Holyoke College
Izower, Jack, City College of New York
Jones, Lynne A., Connecticut College
Krause, Helen, University of Massachusetts
LaFauci, Grace, Wilson College
Mangum, Charlotte, Vassar College
Marzolf, George, University of Michigan
McDowell, Sister Margaret Ann, College of St. Mary of the Springs
McWhinnie, Dolores J., DePaul University
Mescher, Sister Alma L., University of Notre Dame
Moffey, Elizabeth S., University of Michigan
Moulton, John, Hastings College and Clark University
Norbeck, Betty, University of Minnesota
Nordlie, Frank, University of Minnesota
Prosser, Jane Ellen, Earlham College
Rappaport, Lucinda, Brandeis University
Seeck, Margaret A., Oberlin College
Shaw, William N., Bureau of Commercial Fisheries
Shor, Bernice, Rollins College
Simpson, Margaret, Catholic University of America
Sterns, Carol W., Peekskill, New York
Stong, Cynthia C., Wellesley College
Thomas, Caroline, University of Vermont
Verrusio, A. Carl, Drew University
Williams, Junarden, Northwestern University
Zimmerman, William, Princeton University
Zottoli, Robert, Bowdoin College

ECOLOGY

Abbiate, Lorraine M., Douglass College
Bachman, Roger W., University of Michigan
Bianchi, Carla F., Chatham College
Burkholder, K. M., Emory University
Davey, Tessa, Mount Holyoke College
Hayward, George E., Drew University
Palmer, John D., Northwestern University
Pinchot, Gifford B., Johns Hopkins University
McLaughlin, Ellen, University of North Carolina
Sweeney, Edward F., Boston University
Swift, Elijah, Swarthmore College
Taylor, Walter R., Johns Hopkins University
Watt, Walton D., Dalhousie University
Whiteley, George Co., The Hill School
Williams, Elsie Louise, Goucher College

3. Fellowships and Scholarships, 1959

Lucretia Crocker Scholarships:
Charles P. Mason, Botany Course
John D. Palmer, Ecology Course
Conklin Scholarship:
Stephen Levine, Embryology Course
Bio Club Scholarships:
Diana Lee Ehrlich, Botany Course
Jack Izower, Invertebrate Zoology Course

4. TABULAR VIEW OF ATTENDANCE, 1955–1959

	1955	*1956*	*1957*	*1958*	*1959*
INVESTIGATORS—TOTAL	250	304	326	410	427
Independent	162	184	186	203	215
Under Instruction	9	20	23	39	45
Library Readers	54	50	42	54	51
Research Assistants	25	50	75	114	116
STUDENTS—TOTAL	148	140	139	138	134
Invertebrate Zoology	56	55	55	55	49
Embryology	30	28	27	22	23
Physiology	30	30	30	27	27
Botany	19	18	18	18	20
Ecology	13	9	9	16	15
TOTAL ATTENDANCE	398	444	465	548	561
Less persons represented as both investigators and students		2	3	5	4
	398	442	462	543	557
INSTITUTIONS REPRESENTED—TOTAL	129	130	129	142	143
By Investigators	95	97	94	110	98
By Students	34	33	35	74	73
SCHOOLS AND ACADEMIES REPRESENTED					
By Investigators	3	3	5		12
By Students	2	1	1	2	8
FOREIGN INSTITUTIONS REPRESENTED					
By Investigators	8	9	11	20	29
By Students	6	6	5	6	9

5. INSTITUTIONS REPRESENTED, 1959

Abbott Laboratories
A & M College of Texas
Adelphi College
Agricultural Research Center
Alabama, University of
Albert Einstein College of Medicine
American Heart Association
American University
Amherst College
Barnes Hospital
Bowdoin College
Brandeis University
Brooklyn College
Brown University
Bryn Mawr College
Buffalo, University of
California, University of
Carnegie Institution of Washington
Catholic University
Chatham College
Chicago, University of
Cincinnati, University of
City College of New York
Colby College
College of St. Mary of the Springs
College of William and Mary
Columbia University
Columbia University, College of Physicians and Surgeons
Connecticut College
Connecticut, University of
Cornell University
Cornell University Medical School
Cushing Academy
Department of the Interior
DePaul University
Drew University
Duke University
Earlham College
Emory University
Florida State University
Fordham University
Franklin and Marshall College
Georgia, University of
Goucher College
Hahnemann Medical School
Hamilton College
Harvard University
Harvard University Medical School
Hastings College
Illinois, University of
Indiana State Teachers College
Indiana University
Institute for Muscle Research

Iowa State University
Johns Hopkins University
Kansas University
Louisiana State University
Loyola College
Maine, University of
Manhattan College
Marquette University
Maryland, University of
Massachusetts Eye and Ear Infirmary
Massachusetts General Hospital
Massachusetts Institute of Technology
Medical College of Virginia
Michigan State University
Michigan, University of
Minnesota, University of
Montefiore Hospital Research Institute
Mount Holyoke College
Mt. St. Joseph, College of
National Institutes of Health
Nebraska, University of
New Hampshire, University of
New York State University College of Medicine at Syracuse
New York University
New York University, Bellevue Medical Center
New York University School of Dentistry
New York University, Washington Square College
North Carolina State College
North Carolina, University of
Northwestern University
Notre Dame University
Oak Ridge National Laboratory
Oberlin College
Ohio Wesleyan University
Oklahoma, University of
Oregon, University of
Orleans High School
Pennsylvania, University of
Pennsylvania Medical School, University of
Pittsburgh, University of
Princeton University
Purdue University
Queens College
Radcliffe College
Reed College
Research Institute for Advanced Studies
Rhode Island, University of
Rochester, University of
Rockefeller Institute for Medical Research
Rollins College
Roswell Park Memorial Institute
Rutgers University
St. Joseph's College
St. Louis University
St. Mary of the Woods College
St. Peter's College
St. Vincent College
Seton Hill College
Single Cell Research Foundation
Sloan-Kettering Institute
Smith College
Swarthmore College
Sweet Briar College
Syracuse University
Temple University
Texas, University of
Texas, University of, Southwestern Medical School
The Hill School
Tufts University
Tulane University
Upjohn Company
Utah, University of
U. S. Fish and Wildlife Service
U. S. Public Health Service
Vanderbilt University
Vassar College
Vermont, University of
Veterans Administration Hospital
Virginia, University of
Washington University
Washington University Medical School
Washington and Jefferson College
Wayne State University
Wellesley College
Wesleyan University
Western Reserve University
Wilson College
Wisconsin, University of
Woods Hole Oceanographic Institution
Yale University

FOREIGN INSTITUTIONS REPRESENTED, 1959

Instituto de Anatomica y Embriologia, Universidad de Buenos Aires, Argentina
University of Brussels, Belgium
Arcadia University, Canada
Dalhousie University, Canada
McGill University, Canada
McMaster University, Canada
University de los Andes, Bogota, Colombia
King's College, England
Trinity College, England
University College, England
Sorbonne, Paris, France
University of Paris, France
Max-Plank Institüt für Virusforschung, Germany
Technical University, Darmtstadt, Germany

Technische Hochschule, Germany
Madras Christian College, Madras, India
Hebrew University, Israel
University of Milan, Milan, Italy
University of Tokyo, Japan
University of Puerto Rico, Puerto Rico
University of the Philippines, Philippines
Centro Investigaciones Biologicas, Madrid, Spain
Karolinska Institutet, Stockholm, Sweden
Uppsala University, Sweden
Clinique Medicale Universitaire, Switzerland
University of Witwatersrand, Johannesburg, South Africa
Universidad Central de Venezuela, Venezuela
University College of the West Indies, Jamaica, West Indies

SUPPORTING INSTITUTIONS AND AGENCIES, 1959

American Cancer Society
Associates of the Marine Biological Laboratory
Atomic Energy Commission
Josephine B. Crane Foundation
The Grass Foundation
The Lalor Foundation
F. R. Lillie Fellowship
Olin Mathieson Chem. Corporation, Charitable Trust
National Institutes of Health
National Science Foundation
Office of Naval Research
The Rockefeller Foundation
Smith, Kline and French Foundation

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6. EVENING LECTURES, 1959

June 26	R. E. BILLINGHAM	"Studies on the Y chromosome antigen in rodents"
July 3	ALEXANDER FORBES	"The growth of physiology"
July 6	ALEXANDER FORBES	"Electrophysiology of color vision"
July 10	I. M. KLOTZ	"Protein hydration and behavior"
July 17	C. LADD PROSSER	"'The origin' after a century; prospects for the future"
July 24	SILVIO RANZI	"Protein differentiation during embryonic and larval development"
July 31	V. G. DETHIER	"Chemical sense of the blowfly and hunger"
August 7	CLAUDE A. VILLEE	"Interrelations of hormones and enzymes"
August 14	HUGO THEORELL	"Mode of action of enzyme-coenzyme complexes"

August 21	
W. A. H. Rushton	"The retina is the net of a fisherman who catches quanta and barters them for information"
August 28	
Eugene P. Odum	"The energy flow to the study of populations in nature"

7. Tuesday Evening Seminars, 1959

July 7	
J. L. Griffin	"Isolation and chemical identification of the crystalline cytoplasmic inclusions in the large, free-living amebae"
Milton Fingerman	"Physicochemical characterization of chromatophorotropins in the crayfish, *Cambarellus shufeldti*"
William H. Johnson and Andrew G. Szent Györgyi	"The molecular basis for the 'catch mechanism' in molluscan muscles"
July 14	
James D. Eisen	"A study on the physiology of the predator-prey relationship existing between *Paramecium aurelia* and *Didinium nasutum*"
Wolfgang Wieser	"Growth, metabolism and coexistence in marine nematodes"
Ralph A. Lewin	"Uptake of strontium by *Syracosphaera*"
July 21	
A. B. Novikoff	"Lysosomes in the physiology and pathology of cells"
J. R. Collier	"Localization and synthesis of ribonucleic acid in the development of *Ilyanassa obsoleta*"
G. G. Holz, Jr. and C. C. Speidel	"Mating behavior of x-rayed *Tetrahymena pyriformis*." Motion pictures
C. Fulton	"Polarized tissue movement in hydroid regeneration." Motion pictures
July 28	
Philip Person, Jay W. Lash and Albert Fine	"Myoglobin and cytochrome oxidase in odontophore cartilage of Busycon"
W. Troll, S. Belman and N. Nelson	"Aromatic amine metabolism and bladder cancer"
Paul S. Galtsoff and D. E. Philpott	"Ultra structure of the spermatozoon of the oyster"
August 4	
Vincenzo Leone	"Some structures found in electron microscopic pictures of an amphibian tumour"
Alfred W. Senft	"Ultrastructure of the human parasite, *Schistosoma mansoni*"

George W. de Villafranca and Delbert E. Philpott	"A study of the fine structure of skeletal muscle from *Limulus polyphemus*"
August 11	
Maurice M. Rapport	"Present status of the problem of plasmalogen structure"
Eric G. Ball	"On the mode of action of insulin"
Walter S. Vincent and Elyane Baltus	"Incorporation of isotopic label into RNA: synthesis or terminal addition?"
August 18	
L. V. Heilbrunn	"The action of glycerol on protoplasm"
Walter L. Wilson and K. S. Swami	"Electrophoretic studies on protoplasm"
Francis T. Ashton	"Germinal vesicle breakdown in the eggs of Spisula and Hydroides"
R. D. Allen	"Polarized optical studies on Ameba"
F. Child	"Isolation and analysis of cilia"

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V. REPORT OF THE LIBRARIAN

During 1959, forty-eight new journals were acquired making a total of 1665 currently-received titles. Of these, there were 484 (12 new) Marine Biological Laboratory subscriptions, 638 (9 new) exchanges and 184 (5 new) gifts; 100 (7 new) were Woods Hole Oceanographic Institution subscriptions; 199 (12 new) were exchanges and 60 (3 new) were gifts. Between the years 1950 and 1959, 439 new journals were obtained with initial date of publication, in each case, falling within that period.

The Laboratory purchased 92 books (15 of these from the Montgomery Memorial Fund), received 119 complimentary copies (7 from authors and 112 from publishers) and accepted 43 miscellaneous gifts. The Institution purchased 50 books and received 6 as gifts. The total number of books accessioned amounted to 310.

Through purchase, exchange and gift the Laboratory completed 10 journal sets and partially completed 15. The Institution completed 6 sets and partially completed 6. There were 5,629 reprints added to the collection, of which 1772 were of current issue.

At the close of the year there were 76,073 bound volumes and 212,627 reprints.

The Library mailed out on inter-library loan 384 volumes and borrowed 72. About 900 volumes were bound, as well as 85 pamphlets.

Dr. E. V. Cowdry presented his large collection of reprints to the Library, of which 2000 were added to the shelves. Among his collection there were several journal numbers which filled in gaps of long standing. Dr. F. A. Hartman presented a collection which will be processed in 1960. Also, gifts of reprints and books were received from Dr. H. W. Kaan, Dr. P. W. Whiting and Mrs. A. R.

Memhard. Dr. W. R. Amberson donated a long series of the serial entitled "Onderzoekingen gedaan in het Physiologisch Laboratorium der Rijksuniversiteit te Utrecht," as well as several books. To each of these generous friends the Laboratory wishes to extend grateful thanks for the valuable literature acquired by the Library.

Two foreign institutions benefited from the collection of duplicates, namely, the National Institution of Oceanography in England and the Marine Biological Laboratory at Helsingör, Denmark.

During 1959, the Staff noticed a considerable increase in the use of the Library during the winter months. This is very gratifying, as it indicates further year-round use of the Library facilities, as Woods Hole becomes more and more a scientific research center.

Respectfully submitted,
Deborah L. Harlow,
Librarian

VI. GENERAL BIOLOGICAL SUPPLY HOUSE, INC.

It would seem that a short resumé of the history of "Turtox" would be of interest to the members of the Corporation at this time.

In 1913, Morris Wells was a graduate student in the Department of Zoology of the University of Chicago. Dr. Frank R. Lillie was Chairman of the Department, and also President of the Marine Biological Laboratory. Prior to this, Mr. Wells had taught biology for one year in a high school in Kansas. This experience made him realize that biology teachers needed aid in obtaining material for instruction. In 1914, he and his wife prepared a one-page mimeographed sheet, listing slides and other material, which was mailed to a list of biology teachers. Several orders for $1.00 each were received, which were processed in the cellar of Mrs. Wells' parents' home. Mr. Wells received his Doctor's degree in 1915, and accepted a position as Instructor under Dr. Frank R. Lillie, at the University of Chicago. He was promoted to the rank of Assistant Professor three years later. By 1918, Dr. Wells realized he could no longer do justice to his teaching, and take care of his growing business. He discussed this with Dr. Frank Lillie. Dr. Lillie suggested that if Dr. Wells wished to devote all his time to furnishing biological supplies to teachers, he might interest his brother-in-law, Mr. Charles Crane, in the business. A corporation was formed, under the laws of the State of New York; Dr. Frank Lillie was Chairman of its Board of Directors for many years. Mr. Crane purchased 51% of the stock of the new company, and turned it over to the Marine Biological Laboratory as a gift. The amount involved was $10,000.

In 1920, due to the growth of the business, the General Biological Supply House increased their stock by $5000, and the M. B. L. purchased one-half of the new issue for $2500.

In 1921, the Treasurer's report of the M. B. L. shows the entire holdings of General Biological Supply House stock held by M. B. L. listed at $12,700. This figure includes 51% of the voting stock. The purpose of this arrangement was to keep the business in the control of a scientific institution, which would prevent any possible future hazards of the business being run just for the profit of the private owners of the stock. Dr. Wells insisted from the start that the most important functions of the new company were to help biology teachers get material, and pass on to them information concerning new techniques.

The new company was called "The General Biological Supply House Inc." and Dr. Wells coined the word "Turtox," with a design of a turtle holding up the world.

Information about the new company during the first five years is not available, except that 8% of the par value of the stock was paid in dividends during that period. From 1923 to the present date, a period of 36 years, the M. B. L. has received $476,144 in dividends and its stock interest in the company at this time is worth well over $500,000.

In 1919 Dr. Wells had a student in one of his courses who seemed to have potentialities. Dr. Wells suggested that he continue his courses in fundamental biology, and take courses in commerce. This student followed Dr. Wells' suggestion, and took his degree in Business Administration. He worked part time, as a student, for "Turtox," and became a full time employee when he graduated. Dr. Wells was ill for long periods, and this man was elected Vice President. When Dr. Wells died, he became President, a position he still holds. He is, of course, Mr. C. Blair Coursen. Part of Blair Coursen's work until recently was to edit the magazine, "Turtox News"; Mrs. Shepherd, who worked with Mr. Coursen for several years, is the present editor. "Turtox News" devotes more than half its pages to non-advertising material. Secondary school biology teachers find it "good reading."

In 1955, General Biological Supply House moved to a new building, especially designed for their operation. This building has proved to have been a wise investment.

In 1957, the Board of Directors established the "Turtox Scholarship." Any American citizen who is currently, or has been, enrolled in a graduate school of biology is eligible. The award is based upon evidence bearing upon the promise of the applicant as a prospective teacher and research scholar. The stipend is $5000 per year—one of the largest scholarships available. The Scholarship Committee consists of Dr. Frank A. Brown, Jr., Chairman, Dr. Philip B. Armstrong, Dr. C. E. Olmstead, Dr. D. P. Rogers and Dr. S. Meryl Rose.

In preparing this report, I wish to acknowledge help from Mr. C. Blair Coursen, Mrs. Edith Wells, Dr. Winterton C. Curtis, Mr. Homer P. Smith and the Librarian, Mrs. Deborah L. Harlow.

The successful operation of the General Biological Supply House Inc. reflects the management of its President, C. Blair Coursen, ably assisted by the Vice President, Arold Blaufuss, the Export Manager, Charles Coursen, Jr., and the Assistant to the President and Editor of "Turtox News," Mrs. Ruth L. Shepherd. They, together with a group of well trained and loyal employees, have rendered a unique service to teachers of biology throughout the world. Thus, the original purpose of its founder, Dr. Morris Wells—to give aid and assistance to biology teachers—is being carried out.

Respectfully submitted,
C. Lloyd Claff

THE EFFECT OF SALINITY ON GROWTH OF GYMNODINIUM BREVE DAVIS

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Field observations have established the close physical association of mass mortalities of marine animals in the Gulf of Mexico with high concentrations of the non-thecate dinoflagellate *Gymnodinium breve* Davis (Davis, 1948; Galtsoff, 1948, 1949; Gunter *et al.*, 1948; Wilson and Ray, 1956, among others). With the development of satisfactory culture media (Wilson and Collier, 1955) and successful methods for growing the organism in the absence of bacteria (Ray and Wilson, unpublished results), more definitive study of this association became possible. Subsequently Ray and Wilson (1957), and Starr (1958) conclusively demonstrated the toxicity of *G. breve* to fishes.

The catastrophic manifestations of naturally-occurring *G. breve* blooms have attracted considerable attention. The sporadic nature of the outbreaks has stimulated particular interest in possible relationships between environmental factors and these "red tides." In this regard, various investigators, drawing from relatively sparse field data, have postulated the importance of salinity, dissolved nutrients, and meteorological conditions (see Ryther, 1955, for review). This report deals with the effect of salinity on the growth *in vitro* of *G. breve* and compares these findings with the field observations of other workers.

The technical assistance of Mrs. Alice Kitchel is gratefully acknowledged.

MATERIALS AND METHODS

Bacteria-free cultures (10-ml. aliquots in 16 × 125 mm. screw-capped Pyrex tubes) were employed throughout this work. These tubes, together with the flasks and pipettes used in medium preparation or inoculation, were rigorously cleaned before each use. The cleaning routine, found by Ray and Wilson (unpublished results) to be an important factor in the successful culturing of this organism, included the use of a detergent, hot 10% nitric acid, and repeated rinses in tap water and distilled water. In an additional step, culture tubes were filled with, and inoculation micropipettes immersed in, triple-distilled water and autoclaved for 15 minutes at 15 p.s.i.

All control tubes contained a completely synthetic medium (Table I), compounded by one of us (W.B.W.) which supported good growth of *G. breve*. The medium in experimental tubes differed from control medium only in major salt content ($NaCl$, $MgSO_4$, $MgCl_2$, $CaCl_2$ and KCl). In varying salinity these major constituents were varied proportionally, thus producing no change in the ion ratios.

Pasteur capillary pipettes were used to inoculate each of a series of tubes of medium with 100–200 cells from well-established cultures of *G. breve*. Two or three cultures were used to inoculate each experiment. Equal numbers of replicates from each salinity group were inoculated with a given culture so that physiological differences between inocula would not bias results. After inoculation, the new cultures were maintained at a temperature of 26–27° C., and illuminated by two 30-watt "standard cool white" fluorescent lights two to three inches from the culture tubes.

Glassware and media were sterilized by autoclaving at 15 p.s.i. for 15 minutes. After inoculations were completed, two bacterial sterility tests were conducted for each culture used as inoculum. These tests involved pipetting 1 ml. of the inoculum culture into each of two culture tubes, one containing 10 ml. of peptone sea water broth, the other a 10 ml. peptone sea water agar slant (Spencer, 1952).

TABLE I

Gymnodinium breve *culture medium*

NaCl*	29.0 gm.	KNO_2	1.0 mg.
$MgSO_4 \cdot 7H_2O$	6.0 gm.	KNO_3	1.0 mg.
$MgCl_2 \cdot 6H_2O$	4.5 gm.	Thiamine†	1.0 mg.
$CaCl_2$	0.7 gm.	Vitamin B_{12}†	1.0 μg.
KCl	0.6 gm.	Biotin†	0.5 μg.
Tris(hydroxymethyl)-aminomethane**	20.0 mg.	Sulfide solution‡	5.0 ml.
$Na_2S \cdot 9H_2O$	3.0 mg.	Metals solution§	20.0 ml.
K_2HPO_4	1.0 mg.	Triple-distilled water	1000 ml.

* A. R. grade, recrystallized from triple-distilled water by the addition of C. P. HCl. All other inorganic compounds were C. P. or A. R. grade.

** Fisher Scientific Co. Added as 50 ml. of a stock solution adjusted to pH 8.2 by the addition of HCl.

† Nutritional Biochemicals Corp.

‡ Five ml. of this solution (derived from van Niel, 1931) contains: NH_4Cl, 1.0 mg.; $NaHCO_3$, 1.0 mg.; $Na_2S \cdot 9H_2O$, 0.8 mg.; KH_2PO_4, 0.5 mg.; $MgCl_2 \cdot 6H_2O$, 0.2 mg.

§ Twenty ml. of this solution contains: (Ethylenedinitrilo)tetraacetic acid disodium salt (Eastman Kodak Co.), 3.0 mg.; Mn as $MnCl_2 \cdot 4H_2O$, 0.2 mg.; Rb as RbCl, 0.2 mg.; Al as $AlCl_3 \cdot 6H_2O$, 0.1 mg.; Co as $CoCl_2 \cdot 6H_2O$, 0.1 mg.; Cs as CsCl, 0.1 mg.; B as H_3BO_3, 0.1 mg.; Se as H_2SeO_3, 0.1 mg.; Cr as K_2CrO_7, 0.1 mg.; Mo as $Na_2MoO_4 \cdot 2H_2O$, 0.1 mg.; Sr as $SrCl_2 \cdot 6H_2O$, 0.1 mg.; Ti as TiO_2, 0.1 mg.; Zn as $ZnCl_2$, 0.1 mg.; Zr as $ZrOCl_2 \cdot 8H_2O$, 0.1 mg.; Ba as $BaCl_2$, 0.02 mg.; Cd as $CdCl_2 \cdot 2\frac{1}{2}H_2O$, 0.02 mg.; Cu as $CuCl_2$, 0.02 mg.; Fe as $FeCl_2 \cdot 4H_2O$, 0.02 mg.; Ce as $(NH_4)_2Ce(NO_3)_6$, 0.02 mg.; V as NH_4VO_3, 0.02 mg.; Ni as $NiCl_2 \cdot 6H_2O$, 0.02 mg.; Rh as $RhCl_3$, 0.02 mg.; Ru as $RuCl_3$, 0.02 mg.; Sn as $SnCl_2 \cdot 2H_2O$, 0.02 mg.

Growth of the dinoflagellate was estimated by visual examination of the tubed cultures with the aid of a stereoscopic miscroscope, using 9× magnification for most cultures, and 18× or 27× when populations were low. Eleven graded population categories were adopted and "peak population" arbitrarily defined to include the top three. A rough calibration of this method was carried out by making estimates and actual cell counts from the same cultures, and comparing results. This check was conducted on four occasions, and, in all, 99 test cultures were examined by both methods. Cultures showing peak population by visual

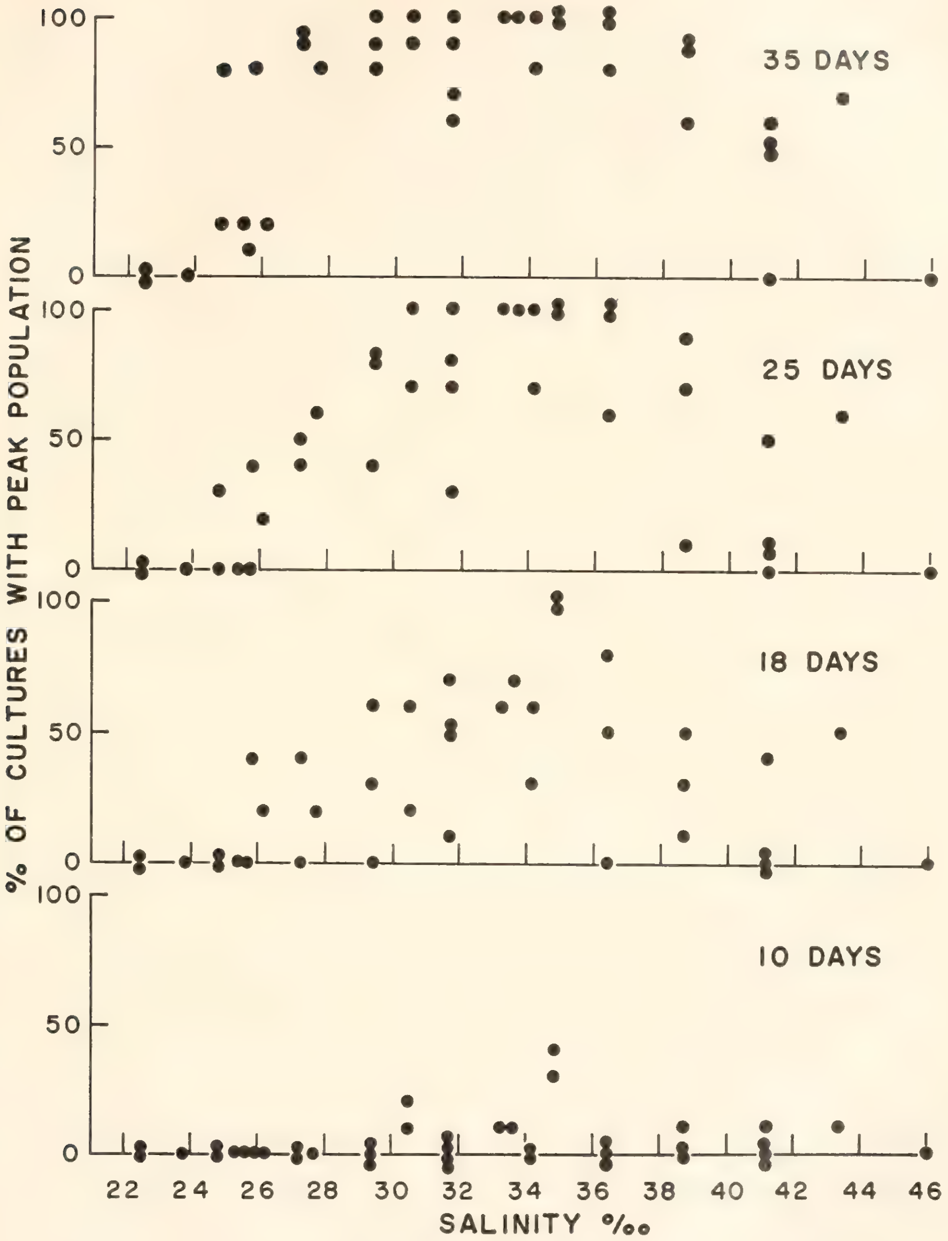

FIGURE 1. Rate of peak population development of *Gymnodinium breve* at various salinities. Each point is based on 10 replicate cultures.

estimate never proved to have fewer than 750 cells per ml., and usually contained from one thousand to several thousand cells per ml. Although sacrificing a degree of quantitative accuracy, the visual estimate method was selected because of the ease, speed, and lack of bacterial contamination with which it could be performed. All cultures were examined by this method at 4, 10, 18, 25, and 35 days after inoculation. The five-week period was considered adequate, since cultures seldom grow after five weeks post-inoculation.

Five experiments were conducted, each having nine salinity levels and ten replicate tubes at each level. The first experiment had the widest salinity range (6.3, 8.4, 11.9, 13.7, 17.8, 18.3, 25.8, 31.7, and 41.1‰). The four later experiments were designed for a salinity range of 22.5 to 41.1‰ with intervals of approximately 2.3‰. However, due to technical error, the last two experiments had ranges of 25.4 to 36.3‰ and 23.8 to 46.0‰ and somewhat irregular intervals.

RESULTS

Cultures of *G. breve* grew well throughout a salinity range of 27 to 37‰ (Fig. 1). Within this range, at least 80% of replicate cultures reached populations of 750 cells or more per ml. during the five-week experimental period. More variable results and generally poorer growth occurred at salinity levels immediately adjacent to the optimal range. No instances of optimum growth occurred at less than 24‰ or more than 44‰. Some indication of comparative growth rates may also be obtained from Figure 1. It is apparent that cultures reach high populations more rapidly within the optimal range (27 to 37‰).

Some organisms survived throughout a salinity range of 22.5 to 46.0‰. From 24.8 to 46.0‰, 91% of cultures contained living cells at the end of the five-week observation period. There was no indication of reduced survival at the extremes of this range. Below this range the incidence of survival was lower; at 23.8‰ the organism survived in only one of 10 replicate cultures, and at 22.5‰ 10 of 20 replicates contained surviving cells. No instances of five-week survival were noted at any of the tested salinity levels below 22.5‰. At 18.3 and 17.8‰ three and two, respectively, of the 10 tubes in each group contained a few live organisms 10 days after inoculation, but no survivors were found eight days later. Media with salt concentrations of 13.7‰ or less contained no visible live *G. breve* one day after inoculation or thereafter.

DISCUSSION

Reports discussing dinoflagellate salinity tolerances indicate a general euryhaline tendency for the group (Biecheler, 1952; Braarud, 1951; Braarud and Rossavik, 1951; Braarud and Pappas, 1951; Nordli, 1953; Provasoli *et al.*, 1954). Of the 10 forms studied by these workers, only one, *Peridinium balticum,* gave evidence of a stenohaline character (Provasoli *et al.,* 1954). Our results suggest that the range of salinity tolerated by *G. breve* is narrower than that of the dinoflagellates studied by other workers.

The literature relating to the occurrence of *G. breve* in the field contains a variety of statements concerning salinity. Fritsch (1956), in a general statement concerning Dinophyceae, noted (p. 664): "In the oceanic plankton the naked types abound, while the neritic plankton is far richer in armoured forms." Commenting on salinities existing during the 1946–47 series of red tide outbreaks along the Florida coast, Gunter *et al.*, (1948) state (p. 320): "Comparing these results with those of normal sea water at various stations in south Florida . . . one may conclude that the . . . salinities are not abnormal." This statement is based largely on salinity values of 35.5 to 37.0‰.

Other workers report the occurrence of *G. breve* blooms in Florida waters having salinities below those associated with the open Gulf. Hela (1956) concluded that 31 to 34‰ represented the most favorable salinity range for blooms. Odum *et al.* (1956) found the organism most frequently in waters having salinities of 33 to 35‰. Observations of *G. breve* blooms by Ketchum and Keen (1948) and Chew (1953) include salinity values with ranges of 32.5 to 33.2‰ and 33.49 to 34.50‰, respectively.

Slobodkin (1953) suggested (p. 151), ". . . red tides require a discrete mass of water of relatively low salinity." He pointed out that the salinity difference between a water mass and the surrounding water tends physically to preserve the identity of the mass. The inference was that the duration of water masses "physiologically suitable" for *G. breve* blooms determined the population levels which could be reached. On the other hand, Ryther (1955), reviewing red tide conditions as reported by Ketchum and Keen (1948) and Chew (1953), pointed out (p. 401) that ". . . where such measurements have been made, the salinity in patches of red water does not appear to be significantly lower than that of the surrounding, clear ocean water."

TABLE II

Salinity and incidence of G. breve *in Florida west coast waters*
(computed from Finucane and Dragovich, 1959)

Salinity range (‰)	Samples collected	Samples positive for *G. breve*		Samples containing *G. breve* in lethal concentrations (250 or more/ml.)	
		Number	Per cent	Number	Per cent
39.00–40.99	107	0	0	0	0
37.00–38.99	1063	27	2.5	0	0
35.00–36.99	4010	397	9.9	15	0.4
33.00–34.99	1473	359	24.4	53	3.6
31.00–32.99	379	55	14.5	1	0.3
29.00–30.99	179	21	11.7	2	1.1
27.00–28.99	114	10	8.8	0	0
25.00–26.99	77	6	7.8	0	0
23.00–24.99	45	4	8.9	1	2.2
21.00–22.99	37	3	8.1	0	0
0.00–20.99	401	0	0	0	0

The obvious differences in the results and conclusions of these field studies are probably related to the small number of observations made in each case. Even so, the noted salinity values fall within the optimum range suggested by our culture studies.

The frequency of *G. breve* occurrence was determined for more than 7000 water samples of known salinity (Table II), using the field data of Finucane and Dragovich (1959). The incidence of the organism was significantly higher in salt concentrations lower (33.00 to 34.99‰) than those usually encountered in waters of the open Gulf (35.00 to 36.99‰). This observation was even more striking when only the potentially fish-killing population densities (approximately 250 or more cells per ml.) were considered.

Reduced salinity *per se* was apparently not a biologically essential factor for good growth of *G. breve* in culture. In the field the association of high incidence of this flagellate with salinities slightly below those of undiluted Gulf water may be due to the presence of dissolved nutrients in land drainage of the Florida west coast (Wilson and Collier, 1955). Water with salt *and* nutrient concentrations conducive to growth of dense populations of this organism may only occur in areas which receive nutrient-rich fresh water. The physical effect of salinity differences on the "life expectancy" of water masses as discussed by Slobodkin (1953) may represent another way in which salinity affects the population of *G. breve*. However, evidence is lacking on this point.

Finucane and Dragovich's (1959) data also show a few instances of the occurrence of *G. breve* at salinity levels between 21 and 25‰. The presence of the organism under these conditions is noteworthy, although the relatively few water samples in this range necessitate wide confidence limits for the per cent incidence values obtained. The long survival of this flagellate in culture at salinity levels inhibitory to growth suggests that it may also exist in the field long after salinity conditions have ceased to be favorable. Furthermore, salinity decreases in the field are probably more gradual than those of our experimental conditions, and may permit more acclimation.

In regard to *G. breve* distribution, our results indicate that high salinity may limit growth of this organism only in areas in which high evaporation with low runoff and mixing cause salt concentrations to rise well above offshore Gulf values. At the other end of the range, however, below 24‰ salinity may be a limiting factor in estuarine environments. Slobodkin (personal communication cited by Ryther, 1955) suggested that between outbreaks "seed populations of this organism are maintained in the brackish to freshwater regions of the Florida Everglades." In opposition to this view, our findings show that marine waters may present the most favorable environment for subsistence. Furthermore, recent field data (Finucane and Dragovich, 1959) indicate a lower incidence of this flagellate in Florida west coast embayments than in the Gulf during non-red tide periods.

When considered with nutritional requirements, the relatively stenohaline character of *G. breve* may explain the comparatively localized distribution of dense populations of this protist.

SUMMARY

1. Bacteria-free *Gymnodinium breve* were exposed to media with salinity values ranging from 6.3 to 46.0‰; the best growth occurred between 27 and 37‰. These results indicate *G. breve* to be a relative stenohaline dinoflagellate.

2. Field evidence associates high incidence of dense populations with salinity levels a few parts per thousand below those of the offshore waters of the Gulf of Mexico. Our results suggest that this field distribution does not represent a salinity requirement *per se,* since salt concentrations equivalent to those of the open Gulf did not inhibit growth of this organism in culture.

3. No instances of optimal growth occurred in culture media with salinity levels of 24‰ or less. Under equivalent estuarine conditions salinity may be a limiting factor in the natural distribution of *G. breve.*

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A MAGNETIC COMPASS RESPONSE OF AN ORGANISM[1]

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Previously reported studies (Brown, Brett, Bennett and Barnwell, 1960; Brown Webb and Brett, 1960) have established beyond reasonable doubt that mud-snails, *Nassarius obsoleta,* are able to perceive and exhibit quantitative alterations in orientational responses to a change in the magnetic field strength amounting to about a 10-fold increase over the earth's natural one. In these studies the experimentally increased field appeared to effect predominantly the same kind of response, whether the field direction remained unaltered or was abruptly rotated through 90° in a horizontal plane. The size of the response to the change in strength of field, and in some measure also the character of the response, were clearly functions of phases of both solar and lunar periods. The response was one of graded amounts of clockwise or counterclockwise turning in the magnetic field.

If orientation in a magnetic field were to be a phenomenon of a useful nature in spatial orientation and navigation, it would be expected that the organism would exhibit a capacity to distinguish the directions of lines of magnetic force as indicated by differential responses to fields at right angles to one another. Suggestion that this might be the case has been reported (Brown, Webb, Bennett and Barnwell, 1959). The following is an analysis to determine whether the snails actually possess such a capacity to respond differentially.

METHOD

The apparatus and methods for obtaining the data have been reported earlier (Brown, Brett, Bennett and Barnwell, 1960). In essence, the experiments comprised permitting snails to emerge from a magnetic-south-directed, straight, narrow corridor into a constant, symmetrical field provided with a grid such that the mean amount of right or left turning during the initial 3 cm. of free movement could be assayed. Each experimental series consisted of two samples of ten passages in the earth's field, two samples of ten in an experimentally increased field oriented as the earth's, and two samples of ten in an increased field rotated 90° clockwise from the natural. The order of the groups of ten in the series of 60 was scrambled except the first three groups of ten in nearly every instance consisted of one with each field, as did also the second of the three groups of ten. Between June 28 and August 29 a total of 564 series of 60 snail runs was obtained between the hours of 5 AM and 9 PM. Though only 17 hours of the solar-day were represented by the data, all hours of a lunar day and all days of a natural synodic month were represented. The number of series for single solar-day hours ranged from 9 to 50, for single lunar-day hours from 5 to 22 and for the single

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days of a natural semi-monthly period (15-day periods synchronized to new and full moon) from 3 to 37.

Results

A daily rhythm was found in the relative effectiveness on the orientation of the snails of the two magnetic fields oriented at right angles to one another. This

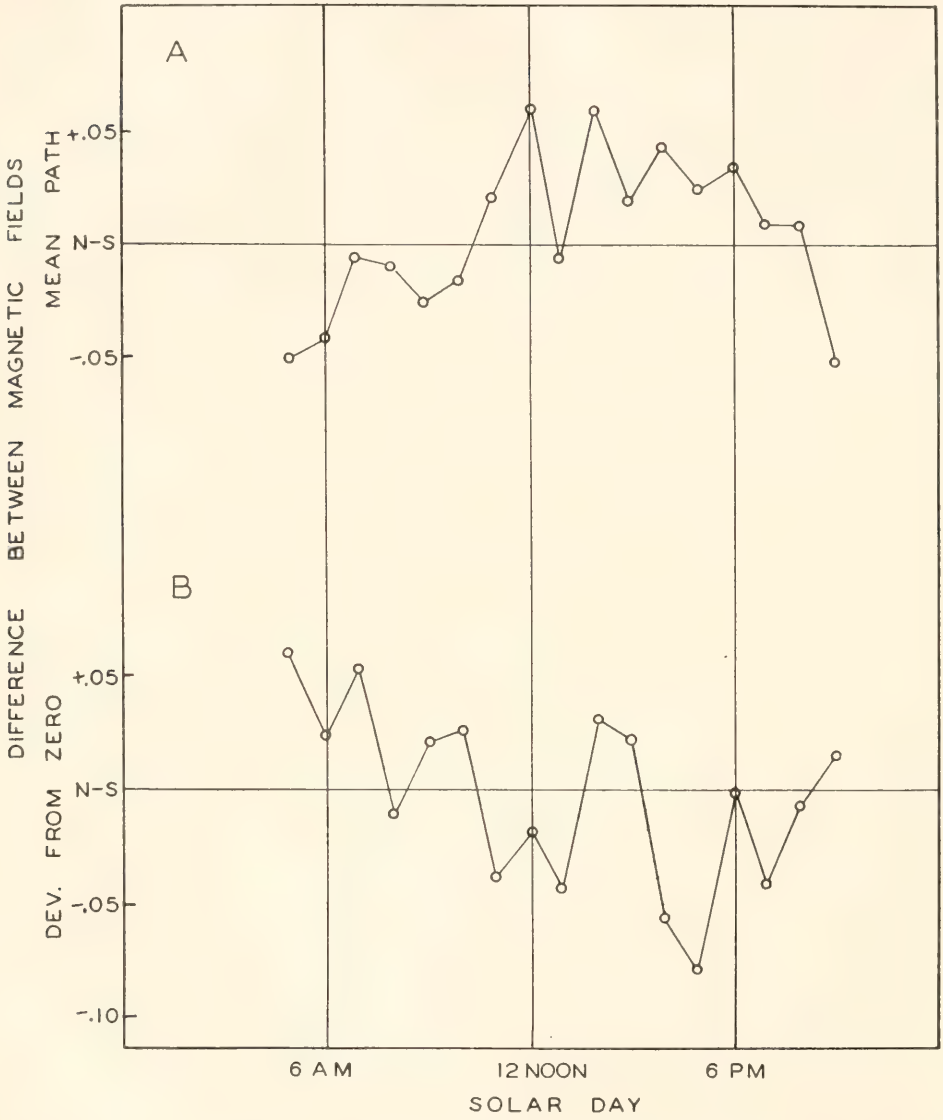

Figure 1. A. The drift through the solar-day of the effectiveness of the E-W magnet field relative to the N-S field in producing mean path difference from controls. B. Same as for A, except for being relative effectiveness in producing dispersion of paths from zero, irrespective of sign.

was demonstrated first for the amount of induced turning of snails in the experimental magnetic fields expressed as difference between the responses of the snails in these fields and the controls in the same series. In Figure 1A is shown the systematic fluctuation in effectiveness of the East-West (E-W) magnetic field relative to the North-South (N-S) field. In the early morning (5–6 AM) the E-W field is the more effective in producing counterclockwise turning of the snails; this is followed by a gradual drift to a time shortly after noon when the N-S field is more effective in producing comparable turning, whereafter, there is a gradual return to a greater effectiveness of the E-W field in the evening. A correlation between the relative effect of the two magnetic fields and the hour angle of the sun expressed as deviation from 2:30 PM (+37.5°) yielded a coefficient of 0.794 ($N = 17$; $P < 0.001$).

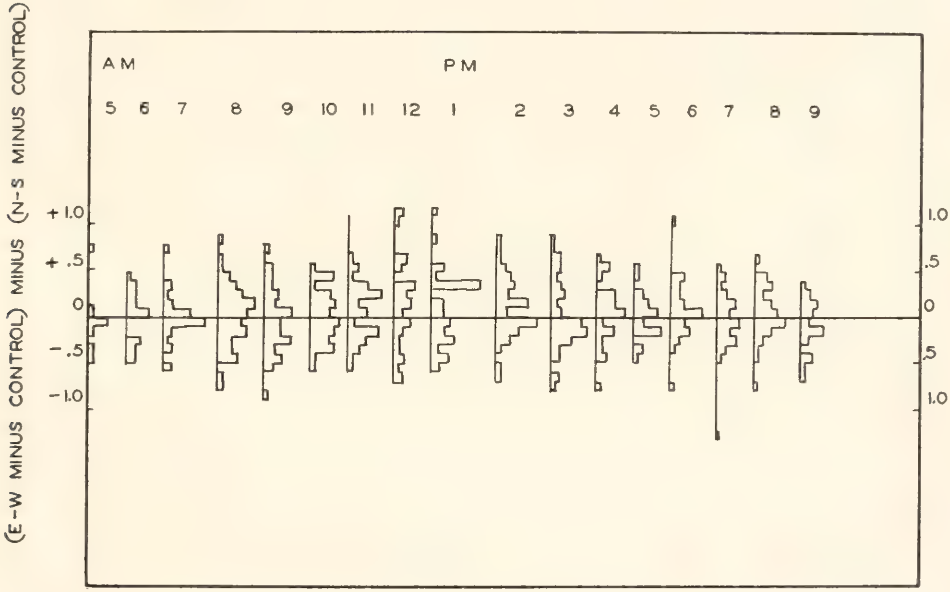

FIGURE 2. Frequency distributions, as a function of hour of day, of the differences between effectiveness of the experimental E-W and N-S fields in altering mean path from the control one.

A comparable daily rhythm was also seen in the difference between the responses to the two magnetic fields in effecting alterations in the total dispersion of pathways, both clockwise and counterclockwise. In Figure 1B it is seen that the E-W field is more effective in producing dispersion of pathways in the early morning, whereas the N-S field tends gradually to become more effective until late afternoon when the relationship seems to reverse again. When this relationship of the two magnetic fields is examined as a linear correlation with the hour angle of the sun expressed as deviation from 4:30 PM (+67.5°), a coefficient of 0.664 is found ($N = 17$; $P < 0.005$).

A daily rhythm in the response as measured by dispersion of paths, or total turning response to magnetic field, whether clockwise or counterclockwise, is perhaps more evident in Figure 2, in which the frequency distributions of the

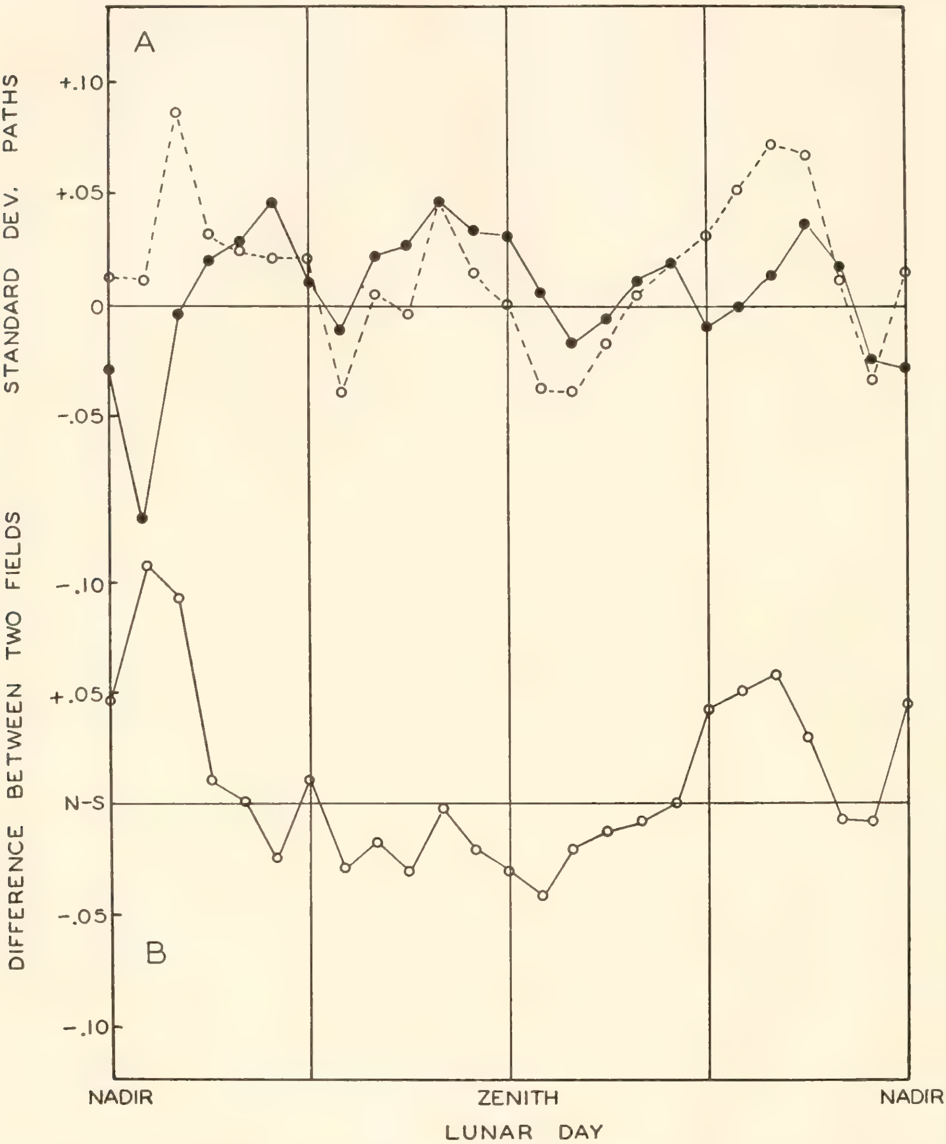

FIGURE 3. A. The difference of the standard deviations for snails in the E-W (dashed line) and N-S (solid line) fields from standard deviation of the controls as a function of hour of lunar-day. B. Drift through the lunar-day of the effectiveness of the E-W field relative to the N-S one in altering standard deviation from that of the controls. Points are calculated from three-hour grouped data.

differences between the responses to the two magnetic orientations are shown as functions of the hours of the solar day. Study of this figure reveals a tendency towards unimodality of distributions in the early morning and evening, with greater dispersion and a definite tendency toward bimodality during the middle of the day. This daily difference between the responses to the two magnetic fields can be demonstrated to be statistically probable beyond reasonable doubt by comparing standard deviations of the distributions. For example, the values for 5–10 AM, 11 AM to 3 PM, and 4–9 PM are, respectively, 0.308 ± 0.0158, 0.389 ± 0.01935, and 0.322 ± 0.0166. The difference between the first and second is 0.081 ± 0.0250 ($t = 3.24$; $N = 394$), and between the second and third 0.068 ± 0.0255 ($t = 2.64$; $N = 391$). As would be expected were this daily rhythm of dispersion a consequence of a mirror-imaging response to the two magnetic fields, differences and their significance can be substantially increased by taking samples for shorter periods symmetrically arranged over the daily period. For example, selecting the three periods, 5–7 AM, 11 AM–1 PM, and 5–7 PM, one obtains values for standard deviations as follows: 0.276 ± 0.0263, 0.413 ± 0.0272, and 0.276 ± 0.0221. Differences between first and second and second and third are, respectively, 0.137 ± 0.0379 ($t = 3.62$; $N = 173$) and 0.137 ± 0.0351 ($t = 3.91$; $N = 196$), differences which are statistically significant beyond all reasonable doubt ($P < 0.001$).

Hence, it is quite evident that the two magnetic fields are being distinguished by the snails, maximally so over the noon hour, and gradually decreasingly so during approximately the preceding and succeeding six-hour periods.

A lunar-day fluctuation in the difference between the dispersion effected by the two positions of the magnet is also evident. This dispersion is indicated here as standard deviation. Using only the 30-day period, July 6-August 4, when the daily series were the most complete and therefore the solar-daily cycle most fully randomized, the relationship between hour of the lunar-day and the magnetic effect expressed as difference from simultaneous control is shown in Figure 3A. The relative effectiveness of the two magnet orientations is plotted in Figure 3B. In view of the differing sizes of the samples for the various lunar-day hours and days of synodic month, these values were calculated as grouped data for overlapping three-hour periods of the lunar day. The results suggest strongly that the E-W field is relatively the more effective during the hours the moon is below the horizon, and the N-S field during the hours the moon is above the horizon. The N-S field is relatively more effective in inducing dispersion just after lunar zenith, becoming progressively less so both as one proceeds to earlier or later hours of the lunar-day. The E-W magnetic field is most effective just after lunar nadir. There is a suggestion of a brief period of greater effect of the N-S field just preceding lunar nadir. This lunar-day cycle displays a qualitatively comparable relationship of the relative effects of the two fields to that seen for the the corresponding hours of the solar day (see Figure 1B).

A very high statistical significance for the lunar-day cycle of relative effectiveness of the two magnetic fields was demonstrated by finding a coefficient of correlation of 0.752 ($N = 20$; $P < 0.001$) between the difference between the two fields and the hour angle of the moon expressed as deviations from –22.5°, through a total of ±11 hours. For this correlation, the individual lunar-day hourly values, of course, were used rather than the three-hour moving means.

However, during a four-hour period of the lunar-day represented by too few experimental series, the data were combined into two two-hour periods, hence the N of 20 instead of the expected 22.

Using data grouped for three-hour periods it was found that there was a complex lunar-day periodism in the difference between the effectiveness of the two magnet orientations in determining mean snail path. This comprised maximum action by the E-W field in causing left-turning one to two hours after lunar-nadir, and maximum left-turning effect of the N-S field between the times of moon-set and nadir (Fig. 4). Superimposed on this last cycle was a lunar-tidal cycle in which a conspicuous, but secondary, increased effectiveness of the E-W field occurs one to two hours after lunar zenith. There is also a suggestion that this bimodal lunar-day, or lunar-tidal, fluctuation in the relative effects of the two magnetic fields possesses still a further, and lesser, harmonic to give a quadri-modal lunar-day cycle.

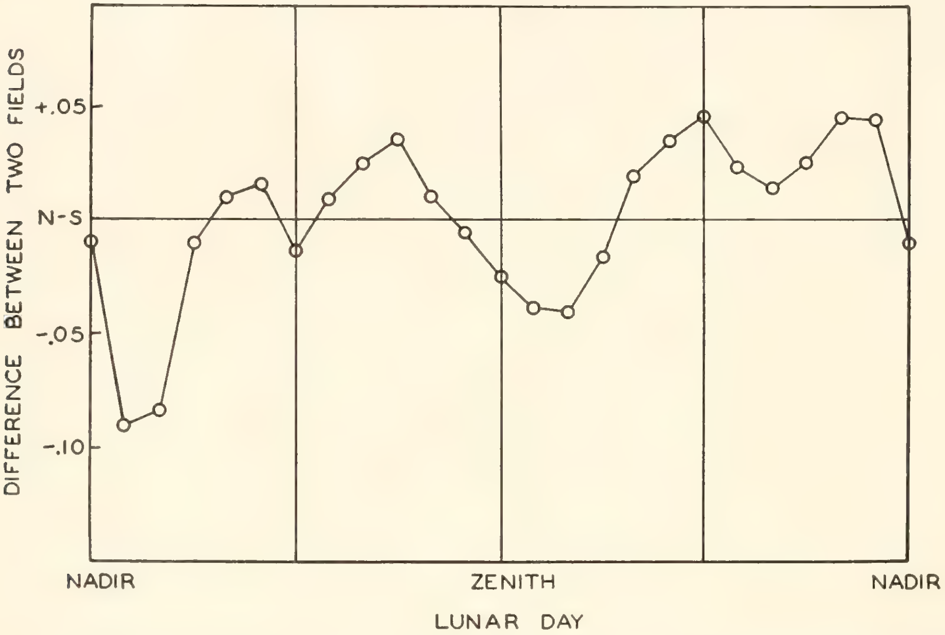

FIGURE 4. The drift through the lunar-day of the effectiveness of the experimental E-W relative to the N-S field in altering mean path from the control one. Points are calculated from three-hour grouped data.

It was shown earlier (Brown, Webb and Brett, 1960) that there was, during the two-month period of study, a semi-monthly cycle of direction and degree of turning of the snails induced by the magnets with maximum clockwise turning a day or two before new and full moon, and maximum counterclockwise turning just before the times of first and third quarters of the moon. When now the actions of the two experimental magnetic fields were compared for their contribution to this semi-monthly rhythm, it was found that the two magnetic orientations

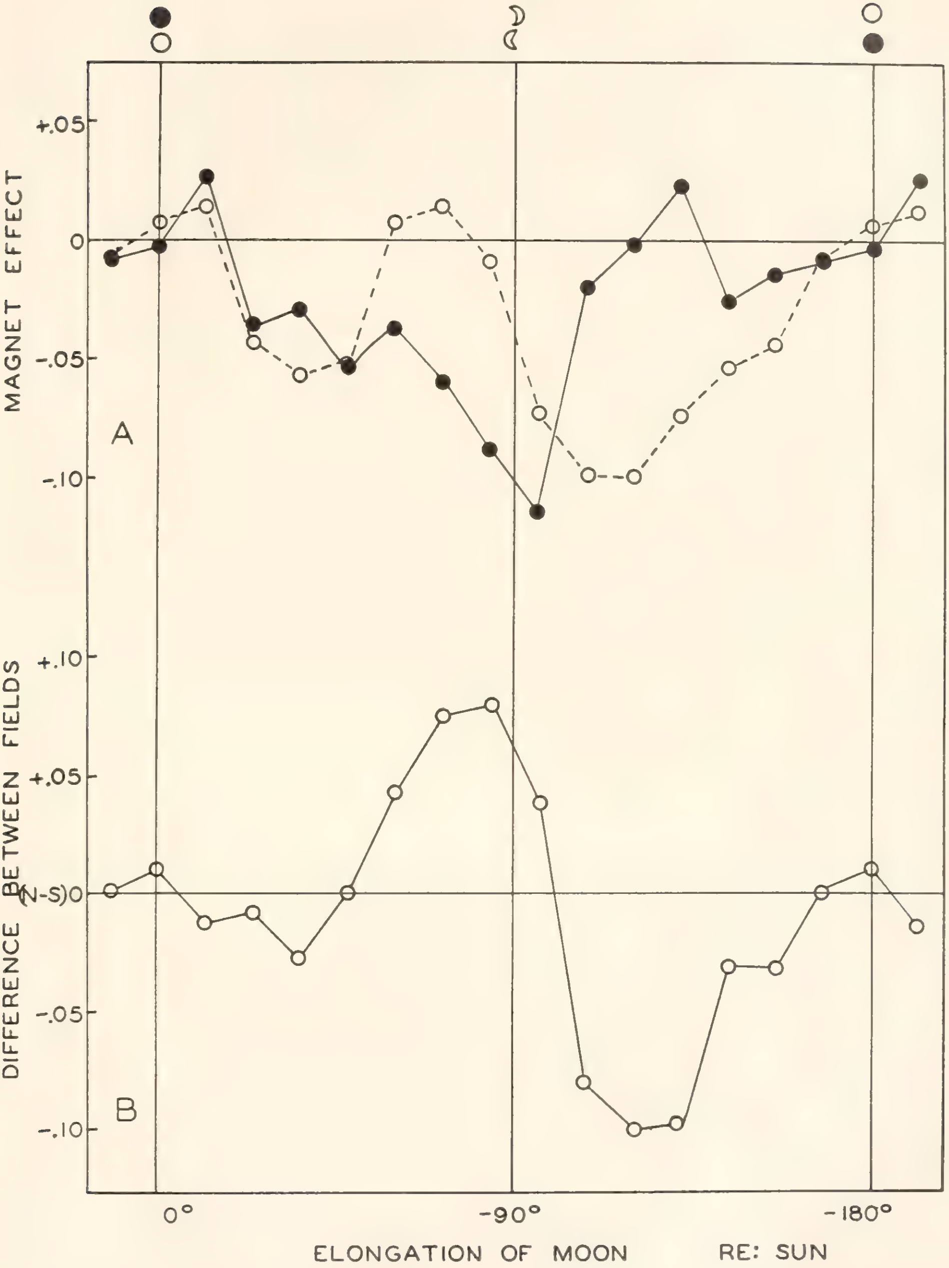

FIGURE 5. A. The drift through the semi-monthly period of the difference between the mean path of snails in the E-W (dashed line) and N-S (solid line) fields from that of the control snails. B. The drift through the semi-monthly period of the effect of the E-W magnetic field relative to the N-S one, in influence on mean snail path. Points are calculated from three-day grouped data.

produced quite strikingly different semi-monthly patterns of effectiveness (Fig. 5A). The difference between them is illustrated in Figure 5B. The E-W field produced relatively a much greater counterclockwise turning about two to four days before the lunar quarters, and then there occurred a very rapid reversal resulting in the N-S field having comparably greater counterclockwise turning action immediately after the lunar quarters. These semi-monthly relationships were computed as overlapping three-day groupings of all data to compensate for greatly differing numbers of series representing the various semi-monthly days.

DISCUSSION

It is evident that the two magnet positions, one with lines of force at right angles to the other, while appearing very similar in effect in indiscriminately pooled data, are in fact not equivalent for the snails at all times of solar and lunar periods. This has been established by the demonstration that the relative effectiveness of the two in inducing left-turning alone, and in affecting total dispersion of pathways (both left and right) exhibit systematic fluctuations through the solar-day, lunar-day and synodic month. In the solar-day, the two fields become essentially equivalent about 8–9 AM and 8–9 PM with difference between the two, in general, increasing systematically during the hours involved in this study as one moves away from these times into the intervening daily hours. For the solar daily cycle, snails moving toward magnetic south in the early morning hours are more strongly turned eastward in an increased magnetic field of E-W orientation than in one oriented 90° to it. On the other hand, by noon, it is the N-S oriented magnet which turns them more strongly eastward. Comparably, in total turning, both eastward and westward, it is the E-W oriented field which produces most turning in the early morning and the N-S field which is most effective from noon to late afternoon. In the daily cycle of response, the snails behave as if they possessed a horizontal directional magnetic axis detector turning through the day like an "antenna."

When the data were rearranged to become lined up in terms of hours of a lunar-day, a quite similar systematic fluctuation was seen, now with the E-W magnetic field exerting the greater turning, either east or west, when the moon was below the horizon, and the N-S field, during the time the moon was above the horizon. This relationship is essentially comparable to the corresponding solar-day one, but the particular phases appear to occur slightly earlier. In this lunar-day fluctuation of the relative influence of the two fields, where all hours of the lunar-day are represented, there is a slight suggestion of a bimodality of the lunar daily cycle with a second, very brief, period of greater effect of the N-S magnet occurring just before lunar nadir.

Although the semi-monthly cycle of the relative effects of the two fields may be at least in some measure an artifact which is a consequence of exclusively daytime sampling, it does lend striking confirmation to the solar-day and lunar-day studies in demonstrating the physiological non-equivalence of the two magnet positions as a function of time.

There seems too little information available to formulate any hypothesis as to how this capacity for magnetic response, so clearly tied in with the fundamental "clock system," might normally operate in orientation of the snail. Orientation in snail populations may occur to some extent by a magnetoklinokinesis, in which the snails would tend on the average to assume a specific orientation at particular times of day as a consequence of different turning responses for different magnetic orientations, relative to the body axis. However, the clear demonstration that snails can also respond to fields by predominantly either left or right turning, again depending upon time, and can differentiate between two magnetic fields, leaves open the possibility that under some conditions, or at some times of their lives, behavioral response of the snails may possess less the character of a klinokinesis and, instead, more that of a clock-regulated taxis, or even a straightforward compass reaction. The demonstration that in one species, there is a clear clock-regulated capacity to orient in a weak magnetic field and to distinguish directions of magnetic lines of force encourages one to postulate that this fundamental capacity may have become associated with highly evolved and specialized mechanisms in species with unusual homing or other navigational capacities.

In the solar-day and lunar-day cycles of difference between the response to the E-W and N-S fields, the snails are behaving quite as if in their response to magnetic field they were to a small but significant extent slaves of magnetic compass needles within their bodies, with these individual compass needles in turn being hands on two kinds of horizontally oriented clocks, solar-day and lunar-day. Some modification of such a compass response might be expected were the well-known "sun-compass" and "moon-compass" orientations of organisms, with their continuous correction for rotation of the earth, dependent in any way upon a continuously altering relationship of the position of these heavenly bodies relative to the direction of the earth's magnetic lines of flux. This might constitute the regulator of the gradually altering sun-compass or moon-compass angle of orientation of the organisms.

It is, of course, common knowledge that to localize the position of the sun or moon which for one or another reason is not immediately visible, it is not sufficient to have only an accurate record of time. One needs an additional parameter, one indicating spatial orientation on the earth's surface. This could not be better nor more directly provided for than through a general organismic capacity for distinguishing direction in terms of the natural magnetic lines. The possession of "living compasses" along with "living clocks" could therefore constitute a potential means for organismic navigation in the absence of more obvious cues.

This demonstrated capacity for differentiating between the directions of the two experimental magnetic fields, since it deals with a weak field strength relatively close to that of the earth's natural one, reinforces still further the earlier conclusions (Brown, Brett, Bennett and Barnwell, 1960; Brown, Webb and Brett, 1960) that the observed solar and lunar daily fluctuations in orientations by the snails in the earth's natural magnetic field and otherwise in a field symmetrical with respect to all previously recognized orienting factors, are at least in part a true orientation in response to that magnetic field.

Summary

1. The snail is able to differentiate between two weak magnetic fields, one oriented at right angles to the other.

2. The relative influence of experimental north-south and east-west oriented magnetic fields in producing dispersion of snail pathways, or effecting a magneto-klinokinesis, displays both solar- and lunar-day rhythms.

3. The relative influences of experimental N-S and E-W fields in effecting a predominantly counterclockwise snail-turning exhibits solor daily, lunar daily, and semi-monthly rhythms.

4. The solar and lunar clock-regulated discriminatory responses for magnetic fields indicate the snail to be significantly oriented as if by internal magnetic compass needles which in turn are hands of horizontal solar- and lunar-day "clocks."

5. Further arguments are advanced for concluding that organismic orientation to the earth's natural weak magnetic field is a normal organismic phenomenon.

6. Implications of this demonstrated solar and lunar "clock-compass" capacity for the well-known "sun-compass" and "moon-compass" orientations of animals are discussed. Also, the insurance value of a "clock-compass" capacity as a potential navigational system for animals when deprived of celestial references, is pointed out.

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THE OCCURRENCE OF β-ALANINE AND β-AMINOISOBUTYRIC ACID IN FLATWORMS

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β-Alanine and β-aminoisobutyric acid were first shown to be products of uracil and thymine metabolism, respectively, in the rat by Fink and co-workers (1956). Recently, the pathway for the formation of these β-amino acids from pyrimidines has been implicated as part of a celluar homeostatic mechanism which is involved in the regulation of ribonucleic and deoxyribonucleic acid synthesis (Canellakis *et al.*, 1959). Since β-alanine and β-aminoisobutyric acid were found to be prominent free amino acids in three species of cestodes (Campbell, 1960a) and have subsequently been shown to arise metabolically from pyrimidines *via* the pathway elucidated in the rat in a fourth species (Campbell, 1960b), the study of the distribution of these two compounds in flatworms was extended to include other symbiotic and free-living species.

β-Alanine, a product of uracil metabolism, was first reported as a decarboxylation product of aspartic acid in bacteria by Virtanen and Laine (1937). It has since been reported to occur in the free state in certain mammalian tissues (Tallen *et al.*, 1954; Roberts *et al.*, 1950), insects (Clark and Ball, 1951; PoChedley, 1956), various plant tissues (Hulme and Arthington, 1950; Synge, 1951), and recently in several species of mollusks (Simpson *et al.*, 1959). β-Aminoisobutyric acid, a product of thymine metabolism, was first isolated from human urine by Crumpler *et al.* (1951). It has since been reported in tissues of the cat (Tallen *et al.*, 1954), the iris plant (Asen *et al.*, 1959), and the mollusk, *Mytilus edulis* (Awapara and Allen, 1959).

MATERIALS AND METHODS

The preparation and extraction of the cestodes was carried out as previously described (Campbell, 1960a). The free-living species and the symbiotic species which have a digestive tract were either kept in the laboratory for a sufficient length of time for the tract to empty normally or this was hastened by applying pressure to the organisms with a glass rod. They were then homogenized and extracted by the method used for the cestodes. Total nitrogen in the extracts was determined by a micro-Kjeldahl method (Lang, 1958). The final solution of the free amino acids was made up to contain one mg. nitrogen per ml. Fifty to 300 microliters of this extract were then spotted on Whatman No. 52 paper for chromatography. Two-dimensional chromatography was carried out by the method of Levy and Chung (1953) with the substitution of a sec-butyl alcohol : formic acid : water solvent system (Hausmann, 1952) in the first dimension. The papers were developed two times in the first dimension. The lowest limit of detection for these two compounds is approximately 0.05 micromoles; consequently, a concentration of β-alanine or β-aminoisobutyric acid in the extracts of less than 0.05 micromoles per 300 microliters extract would not be detected. After the initial chromatography indicated that β-alanine and/or β-aminoisobutyric acid were present, the following methods were used to obtain chromatographic evidence for the identity of these two compounds. In most cases the free amino acid extracts of the flatworms which contained β-alanine and/or β-aminoisobutyric acid were further separated into a basic, neutral, and acidic fraction with Amberlite CG-50 H^+ and Dowex 50 H^+ (Simpson *et al.*, 1959). The neutral fraction contained the β-amino acids. Known β-alanine and β-aminoisobutyric acid added to this fraction or to the extracts gave no additional spots upon two-dimensional chromatography and there was an intensification of the unknown spots in the extracts which were thought to be β-alanine and β-aminoisobutyric acid. The β-amino acids were then separated from the remainder of the amino acids by streaking the neutral fraction or the extracts on Whatman No. 3 MM paper, chromatographing the papers one-dimensionally in sec-butyl alcohol: formic acid: water two times, and eluting the appropriate portion of the papers to recover the β-amino acids. These eluates, which contained a single β-amino acid, were then concentrated and run in several solvent systems with and without added known β-alanine and β-aminoisobutyric acid. These known compounds were also run as reference compounds by themselves. Identical R_f values were obtained with five solvent systems for both the known compounds and the compounds isolated from the worms. These data are given in Table I. Additional evidence for the nature of the compounds was obtained by pre-treatment of the paper with alkaline copper carbonate. This treatment did not hinder the movement of these compounds during chromatography. Crumpler and Dent (1949) have shown the α-amino acids are rendered immobile as far as chromatography is concerned by the formation of a copper complex, whereas the R_f values of β-amino acids remain relatively the same in the presence of copper.

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TABLE I

R_f values of known β-alanine and β-aminoisobutyric acid and compounds isolated from flatworms

Solvent system	R_f	
	β-Alanine	β-Aminoisobutyric acid
(1) sec-butyl alcohol:formic acid:water (75:15:10, by vol.; developed 2x)	0.52	0.67
(2) m-cresol:phenol:borate buffer, pH 8.3 (30:15:7.5, w/w/v)	0.41	0.53
(3) phenol:water (72:28, v/v)	0.66	0.76
(4) lutidine:water (62:38, v/v)	0.30	0.35
(5) methyl alcohol:pyridine:water (80:4:20, by vol.)	0.49	0.58

Whatman No. 52 paper (solvents 1 and 2) and Whatman No. 3 MM paper (solvents 3, 4 and 5)

Results and Discussion

The results of this survey are presented in Table II. β-Alanine and β-aminoisobutyric acid have been identified as free amino acids in all the species of cestodes thus far examined. In *Hymenolepis diminuta,* the concentrations of β-alanine and β-aminoisobutyric acid are approximately equal, amounting to 0.60 ± 0.04 and 0.65 ± 0.08 micromoles, respectively, per gram of tissue (determined by the method of Fowden, 1951). On the basis of the relative intensities of the ninhydrin reaction, β-alanine occurs in higher concentration than β-aminoisobutyric acid in the other four species of cestodes. In *Macrapis cristata,* β-aminoisobutyric acid is in higher concentration than β-alanine. In *Bdelloura candida* and *Entobdella bumpusi,* the two are approximately equal. In this survey only two strictly free-living species were examined, consequently no generalizations can be made concerning the occurrence of these two compounds in symbionts *vs.* free-living species. However, these data indicate that β-alanine and β-aminoisobutyric acid are quite common free amino acids in symbionts which are in contact with host tissue. A notable exception in this case is the liver fluke, *Fasciola hepatica,* which does not have either β-amino acid. It has been shown (Campbell, 1960b) that *H. diminuta* degrades uracil and thymine to β-alanine and β-aminoisobutyric acid, respectively, *via* the intermediate dihydropyrimidines and carbamoyl-β-amino acids which were first shown to be intermediates in rat liver (Fink *et al.,* 1956). Since this path-

Table II

The distribution of β-alanine and β-aminoisobutyric acid in flatworms

Species	Habitat	β-Alanine	β-Aminoisobutyric acid
Turbellaria:			
Bipalium kewense	Free-living (land)	–*	–
Dugesia tigrina	Free-living (fresh-water)	–	–
*Stylochus zebra***	Symbiotic with hermit crab (*Pagurus*)	–	–
Bdelloura candida	Symbiotic upon horseshoe crab (*Limulus*)	+*	+
Trematoda:			
Fasciola hepatica	Endoparasitic in liver of cattle	–	–
*Fascioloides magna***	Endoparasitic in liver of cattle	+	–
Macraspis cristata	Endoparasitic in liver of sting ray (*Dasyatis centrura*)	+	++
Entobdella bumpusi	Symbiotic upon sting ray	+	+
Cestoda:			
Hymenolepis diminuta	Endoparasitic in gut of rat	+	+
Calliobothrium verticillatum	Endoparasitic in gut of dogfish shark (*Mustelus canis*)	++	+
Lacistorhynchus tenuis	Endoparasitic in gut of dogfish shark	++	+
Phyllobothrium foliatum	Endoparasitic in gut of sting ray	++	+
Disculiceps pileatum	Endoparasitic in gut of dusky shark (*Carcharhinus obscurus*)	++	+

* + denotes that the compound was present; – denotes that it could not be detected; ++ denotes that this compound occurs in higher concentration.

** Only one specimen available for analysis.

way may also be present in the other species of flatworms which have these β-amino acids, the presence or absence of these compounds may reflect the availability of pyrimidines or pyrimidine-containing compounds in the environments of the worms. Another possibility is that the species which do not contain β-amino acids as free compounds are incapable of degrading uracil and thymine. This latter possibility is not known at the present.

The author would like to express his appreciation to Drs. John S. Laurie, Clark P. Read, John E. Simmons, and Paul P. Weinstein for their assistance in collecting and identifying the flatworms used in this investigation.

SUMMARY

A survey of thirteen species of free-living and symbiotic flatworms for β-amino acids has been carried out. β-alanine and β-aminoisobutyric acid were found to occur most commonly as free amino acids in the symbiotic species in contact with the tissues of their host. These compounds were not detected as free amino acids in the two free-living species which were examined.

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THE FEEDING MECHANISM IN THE SAND DOLLAR MELLITA SEXIESPERFORATA (LESKE)

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MacGinitie and MacGinitie (1949) give the following account of feeding in the scutellid echinoderm *Dendraster excentricus* (Eschscholtz) (p. 237): "The spines on the upper side of the sand dollar are club shaped and are covered by cilia. These cilia create currents that flow from the direction in which the animal is moving toward what could be called the posterior edge. . . . As the currents flow through these spines, little eddies are created at the posterior sides of the spines. These eddies allow tiny particles and organisms to become trapped in mucus that is secreted onto the surface of the spines. This mucus goes downward and is led into tiny tracts to unite with others. These in turn unite again, passing around the edge to the underside, until near the mouth five tracts or strings of mucus feed directly into the mouth of the sand dollar." This appears to be the only available account of feeding in the Clypeasteroida. Hyman (1958) studied the five-lunuled sand dollar *Mellita quinquiesperforata* (Leske), but was unable to elucidate the feeding mechanism. The present paper describes observations made on another of the lunuled sand dollars, *Mellita* (*Leodia*) *sexiesperforata* (Leske), and shows that it is a ciliary mucus feeder collecting particles on the aboral surface and transporting them through the lunules and around the margin of the test to food tracts on the oral surface.

M. sexiesperforata is common in certain shallow water sandy areas around Jamaica and normally lives either in the surface layer of the sand, so that its outline is discernible from above, or else buried in the sand very close to the surface. The animals used for these observations were collected on the Port Royal Cays and observations were made both in the aquarium and in dishes under a microscope.

MORPHOLOGY

Figure 1 shows an individual in both oral and aboral views. The size of the animals varies considerably, fully grown animals being about 70 to 80 mm. in diameter. In surface view the animal has a roughly pentagonal outline and its surface is pierced by six slit-like lunules: five of these are in the ambulacral areas, the sixth or anal lunule is interambulacral and marks the posterior side of the animal. The anterior side of the test is markedly pointed. Both aboral and oral surfaces are densely clothed with short spines which are described below. On the aboral side of the only other structures visible are lunules, petaloids, and gonopores. On the oral surface the mouth is central and the anus lies posterior to it, just on the edge of the anal lunule. Leading away from the oral margins of the five ambulacral lunules are a number of broad *food tracts*: one of these, the radial tract, leads straight from the inner tip of the lunule to the mouth, the remainder run laterally from the lunule and terminate in the *ambulacral* or *food grooves.* These are deep but narrow grooves, a pair to each ambulacral area running into the mouth; the two members of a pair form a petaloid outline and unite just before reaching the mouth. The food tracts of the anal lunule are less well developed and discharge laterally to the ambulacral grooves of the two neighboring lunules.

Figure 2 shows the profile of a sagittal section through the test and shows that it is thin around the margin and dome-shaped in the center; the anterior margin is thicker than the posterior margin. The mean measurements for the ten individuals of 60 to 65 mm. diameter are:

Anterior margin:	1.8 mm.	±0.04
Central dome:	5.9 mm.	±0.49
Posterior margin:	1.0 mm.	±0.01

It is significant that the animal progresses through the sand with the anterior and thicker margin foremost.

Podia

On the oral surface there are dense concentrations of podia around the periphery of the test, around the margins of the food tracts and in the lunules. They are less dense on the remainder of the ambulacral areas and are absent from areas

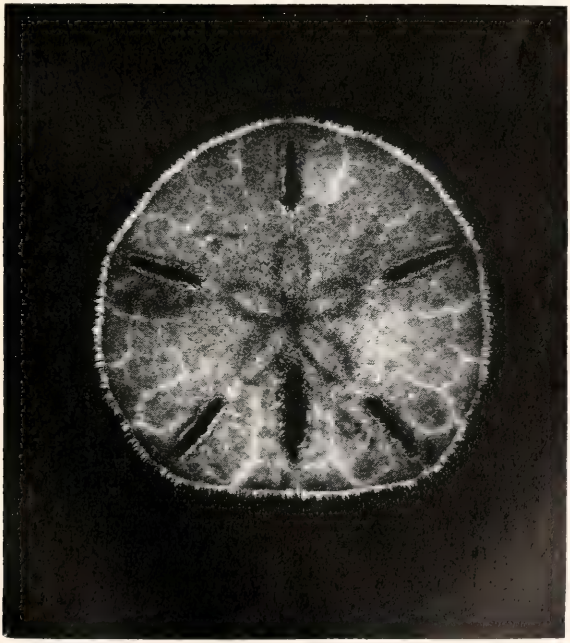

FIGURE 1, a. The aboral surface of a living *Mellita sexiesperforata.* The anterior end is towards the top of the picture. The club-shaped spines appear as minute spots all over the surface of the animal. Notice that the peripheral (ambulatory) spines are more dense anteriorly than posteriorly. The ring of protective spines can be seen clearly around the margin of each lunule.

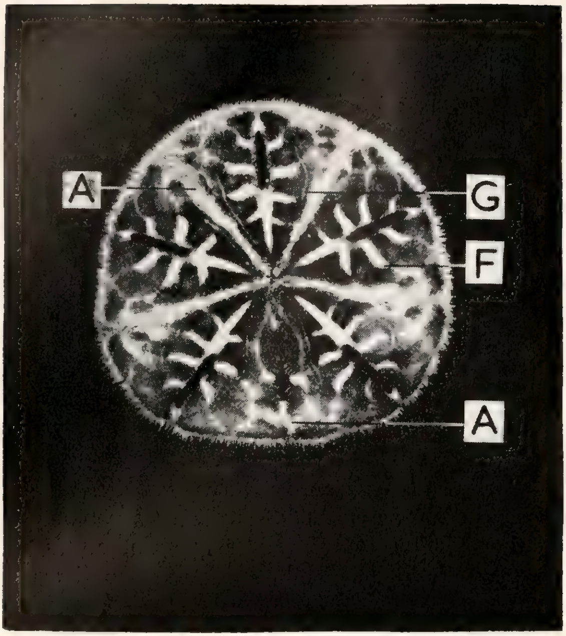

FIGURE 1, b. The oral surface of a living *Mellita sexiesperforata* stained for two minutes in toluidine blue. Orientation as in Figure 1, a. The stain is taken up by podia and other structures, and was used to improve contrast. The mouth is central and the anal papilla appears as a black spot between it and the anal lunule. The anal spines between anus and mouth are just visible. A = Ambulatory spine; F = Food tracts; G = Food (ambulacral) grooves.

with ambulatory spines. On the aboral surface there are only a few scattered podia. When extended the podia are very long and thin with a slightly swollen tip and poorly developed sucking disc. They function exclusively as an accessory food collecting device (see below) and do not appear to play any part in locomotion.

Spines

There are four principal types of spine. On the oral surface ambulatory and non-ambulatory spines can be distinguished. Ambulatory spines are confined to five locomotory areas in the interambulacra of the oral surface. In the anal interambulacrum they form a transverse group behind the anal lunule, in the other four interambulacra they form a radially disposed wedge-shaped group (Fig. 1b). These spines, which are long and thick ($1300\,\mu \times 85\,\mu$) normally have a rounded tip but in many spines the tip is abraded into a roughened end (Fig. 3, A); their

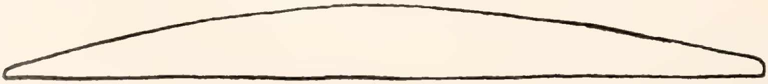

FIGURE 2. Profile of *Mellita sexiesperforata*. The anterior end is toward the right of the picture.

function appears to be entirely that of locomotion. Similar spines are found all around the margin of the test but they are almost twice as thick ($1300\,\mu \times 160\,\mu$); they are densely arranged anteriorly and more scattered posteriorly. Their extra thickness is probably correlated with stresses set up during movement through the sand in a horizontal direction. Non-ambulatory or protective spines of the oral surface are shorter and thinner ($430\,\mu \times 30\,\mu$) than ambulatory ones and are often bent near the middle of their length (Fig. 3, B); they cover all the remaining areas of the oral surface. Similar spines are found in the lunule and on the aboral margin of the lunule they form a protective ring projecting up higher than the other spines of the aboral surface; here they prevent very large sand particles from entering the lunule and blocking it.

On the aboral surface there are two types of spine distributed together all over the animal. The larger of these are club-shaped spines which move the sand grains posteriorly over the aboral surface (Fig. 3, C). The club-shaped head of these spines is set at a slight angle to the stem and its tip is oriented toward the margin of the test. The others or miliary spines (Fig. 3, D) are shorter than the club-shaped spines and are characterized by having a sac-like swelling on the tip; this sac contains yellowish granules which stain darkly with toluidine blue. The epithelium at the base of the cilia on these spines stains purple with the same stain and it seems plausible therefore to suggest that these spines are the principal site of mucus secretion. Miliary spines also occur on the inner walls of the lunules and along the food tracts of the oral surface; the latter have a smaller sac than those of the aboral surface or lunule.

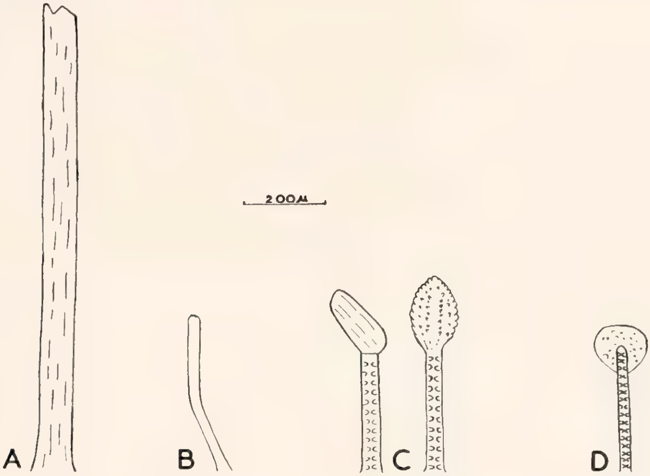

FIGURE 3. The principal types of spine found in *Mellita sexiesperforata*. A = Ambulatory spine with abraded tip. B = protective spine of oral surface. C = Two views of club-shaped spines of aboral surface. D = Aboral miliary spine.

As well as these four main types of spine there is a group of very large spines forming a circle between the mouth and anus and with their tips overarching the anus. They play an important part in preventing feces from entering the mouth.

These spines are in three groups: seven or eight very long ones (up to 5 mm. in length) form a group nearest to the anus, outside these there are ten to twelve of intermediate size, then a group of many smaller ones forming an outer ring next to the mouth. (Fig. 1b).

Cilia

Cilia are confined to the epithelia of the spines and are found on only one side of the stem. This side of the stem is here referred to as its back and the cilia beat in such a way as to drive a horizontal current from it in a backward direction. On the aboral surface the club-shaped spines have dense ciliation at the base while the miliary spines have dense ciliation along the whole length of the stem. The club-shaped and miliary spines are oriented in such a way as to produce centrifugal currents across the aboral surface leading away from the center and towards the margins and lunules. On the oral surface the ambulatory spines have a thin ciliation at the base while the non-ambulatory spines have dense ciliation at the base producing a strong current. The ciliation of the miliary spines of the food tracts is similar to aboral miliary spines. At the margins of the oral surface the spines are oriented so as to drive currents in towards the center. In the food tracts they produce currents towards the mouth in the radial tracts and laterally towards the ambulacral grooves in the remainder. Over the rest of the ambulacral areas the currents drive towards the adjacent ambulacral grooves.

Feeding Behavior

M. sexiesperforata is a microphagous feeder. Removal and microscopal examination of the food cord passing along the ambulacral groove shows that most particles are less than 20 μ in diameter and in a high proportion of them are of the order of 1 μ in diameter. Within these size limits there appears to be no selection of different types of food—algal cells, detrital particles and sand grains are all collected. Larger particles are sometimes collected and sand grains which only just fit in the groove have been seen passing along it and entering the mouth.

An active animal ploughs slowly through the surface sand always keeping the anterior end (Loven's Ray III) forward. As it moves it builds up a small wall of sand in front of itself, the sand grains of which constantly fall down on the aboral surface where they are carried slowly backwards on the tips of the club-shaped spines. This movement of sand appears to be oriented posteriorly across the aboral surface without special reference to the lunules, but small sand grains which reach the margins of the lunules are carried down through them; larger particles are carried off the posterier margin of the test. Particles entering the lunule usually do not drop through it, but are lowered slowly down by means of the spines and then deposited back in the substratum; during this time they may be actively probed by podia in the lunule, suggesting that the latter may remove from it minute absorbed particles.

If carmine particles instead of sand are placed on the aboral surface of the animal a further process of selection may be observed. Larger particles are treated exactly as sand and it can also be seen that the very small particles (visible only under the microscope) drop down between the club-shaped spines and are carried away by the ciliary currents around the spines. At this lower level some particles travel to the margin of the test and then around it to the oral surface, other particles are carried to the lunules and pass down through them and onto the food tracts on the oral surface. By placing carmine particles in the food tracts of an inverted animal it can be shown that the ciliary currents lead to the ambulacral grooves, except in the radial tract where they lead directly to the mouth. Carmine particles placed on the aboral surface of an animal appear in the ambulacral grooves in a few moments. In the ambulacral groove food can be seen to be loosely aggregated in mucus but the precise point at which the mucus is secreted is still in doubt. As pointed out above the miliary spines of the aboral surface are the most probable site of mucus secretion. In the ambulacral groove the food is carried along by podia and not by cilia.

In summary, then, an animal ploughing through the sand pushes sand onto the aboral surface where it is crudely sorted, large particles being carried along on the tips of the club-shaped spines and ultimately deposited back in the sand either through the lunules or off the posterior edge of the test. Fine particles fall down between the bases of the spines where they are carried away by ciliary currents through the lunules to food tracts on the oral surface. From the food tracts cilia carry them to the ambulacral groove thence to the mouth in a mucus aggregation carried along by podia.

There remains the question of food collection by the podia. Because they are confined to the oral surface it is difficult to see them in normal function, but following the method of Nichols (1959) I have examined them in a perspex box with the aid of a prism and a binocular microscope. Under these circumstances the podia at the margin are seen to be constantly extending and contracting and probing the surroundings of the animal. If carmine particles or yeast stained in congo red are pipetted around the margin, particles may sometimes be seen to be picked up by the podia and drawn in to the margin of the animal where they are released and carried inwards by the ciliary currents. At the same time many podia are seen to extend and contract with no visible particles attached. However, only relatively small magnifications (× 30) can be used successfully in examining with the prism and I believe that the principal function of these podia is to probe the surrounding sand for very small particles of food, *i.e.*, particles of about 1 μ diameter. Mention has already been made of the manner in which sand grains are probed by podia as they pass down through the lunules. In the perspex box only a few scattered grains of sand can be included with the animal; otherwise they obscure it from view. The animal thus rests directly on the bottom of the box and only the marginal podia, which extend laterally, can be extended effectively. It seems certain, however, that under normal conditions the podia of the oral surface all probe the sand in the same way, collecting small food particles.

DEFECATION

In an animal such as *M. sexiesperforata* in which the anus is in close proximity to the mouth, special precautions are required to ensure that feces do not re-enter the mouth. Although many animals have been examined from time to time defecation has only been observed on a very few occasions and it appears that it must be an intermittent and not a continuous process.

Defecation commences with a pumping action of the anal papilla followed by cessation of feeding activity; passage of the mucus cord in the ambulacral groove stops completely. The spines between the mouth and the anus beat gently away from the mouth and over the anus, and all around this area pedicellariae become intensely active. The tip of the anus is directed towards the anal lunule and feces are ejected in intermittent puffs of loose particles and not in a mucus string. Examination of a defecating animal from below shows that feces are not, as might be expected, carried up through the anal lunule and so removed in water currents. They fall down from the animal and must in normal circumstances be deposited back in the sand in which the sand dollar community is feeding.

Grateful acknowledgment is made to the Nuffield Foundation from whom the author was in receipt of a research grant when these observations were made.

SUMMARY

1. A brief description of *Mellita sexiesperforata* is given and the process of food collection and defecation are described.

2. Sand pushed onto the aboral surface is sorted by club-shaped spines. Fine particles drop down between the spines and are carried round to the oral surface by ciliary currents, thence to the ambulacral grooves. Mucus is probably secreted by the miliary spines on the aboral surface; podia play an accessory role in food gathering.

3. Defecation is an intermittent process and feeding stops while it is in progress. Spines and pedicellariae prevent feces from reaching the mouth.

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CLEAVAGE WITH NUCLEUS INTACT IN SEA URCHIN EGGS

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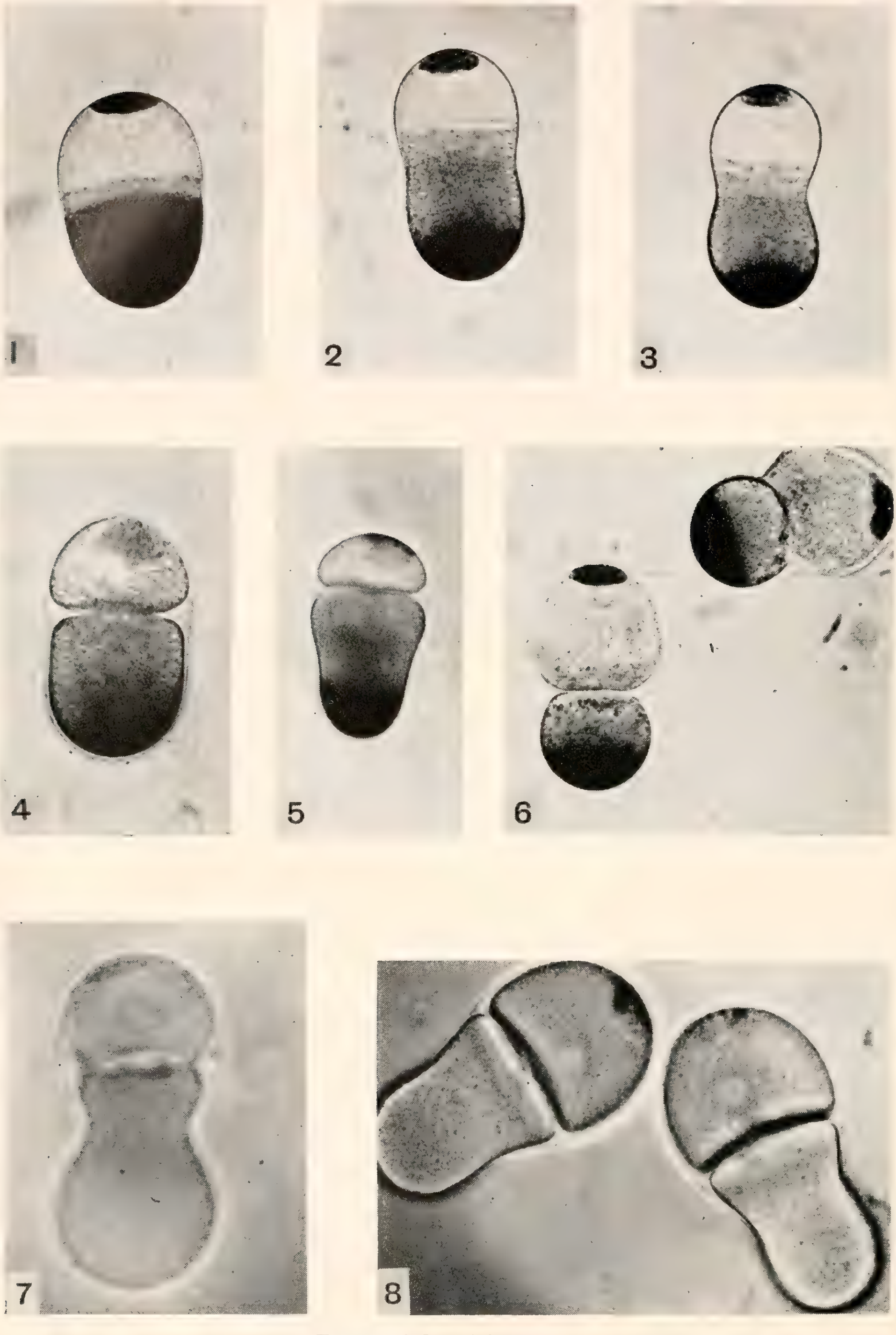
Though, in general, nuclear changes associated with mitosis precede the cleavage of a cell, there are some rare cases in which this is not true. It has been found that in some cases in developing sea urchin eggs, the nucleus may remain as it is in a resting cell, but nevertheless the cell may cleave as it does after mitosis, and produce two quite normal "resting" cells. This has been found to occur in centrifuged eggs which have been stimulated to develop parthenogenetically by treatment with hypertonic sea water (30 grams of NaCl per liter of sea water) for 5 to 15 minutes. After one half to one hour, the egg nucleus remains unchanged but a cleavage plane may come in between the nucleate part of the cell and the non-nucleate part, as it does in such eggs when fertilized (see E. B. Harvey, 1932), resulting in two cells. This has been found to occur in *Sphaerechinus granularis, Psammechinus* (*Parechinus*) *microtuberculatus* and more recently in *Arbacia punctulata* and *A. pustulosa* (photographs of living eggs are reproduced in Figs. 1–8). These occasional cases have been observed over a period of twenty years. No further development or change has been observed. It has not been possible to produce such a cleavage lacking nuclear change, with any of many chemical substances tried.

There are in the literature a few references indicating that the nucleus may be removed experimentally. Mazia and Dan (1952) succeeded in removing the mitotic apparatus in an isolated condition from the "fixed" *Strongylocentrotus franciscanus* egg, and later Dan and Nakajima (1956) removed it "fixed" from other sea urchins, *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus*, with observations also on *Arbacia punctulata*. According to Swann and Mitchison (1953), the eggs of the heart urchin, *Clypeaster japonicus*, may be treated with concentrated colchicine at mid-anaphase, completely abolishing the asters and spindle, and still the egg will divide. There is, of course, the possibility that some part of these structures still remains. To make the experiment more decisive, Hiramoto (1956) sucked out the spindle and asters with a micropipette inserted into the egg, and he found that cleavage still took place.

Some years ago (1938), I made a reference in one of my papers to "cleavage planes coming in independently of any nuclear changes" (p. 182) in sea urchin eggs, and Holtfreter called attention to this in his 1948 paper (p. 723). My paper was accompanied by photographs (44 and 57, 58).

SUMMARY

There now seems no doubt that cleavage can take place without any visible change in the nucleus.



FIGURES 1–8. Living eggs.

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FIGURES 1–3. Stratification of unfertilized eggs of *Arbacia punctulata* with centrifugal force.

FIGURES 4–6. Cleavage of centrifuged, parthenogenetic eggs of *Arbacia punctulata,* without the nucleus taking part. Notice the nucleus in Figure 4, slightly enlarged. It becomes sometimes, not always, slightly enlarged.

FIGURE 7. Cleavage of parthenogenetic, centrifuged eggs of *Sphaerechinus granularis,* without the nucleus taking part.

FIGURE 8. Cleavage of parthenogenetic, centrifuged eggs of *Psammechinus* (*Parechinus*) *microtuberculatus* without the nucleus taking part.

DEVELOPMENTAL STAGES OF THE BROAD BREASTED BRONZE TURKEY EMBRYO[1], [2]

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In studying early mortality in the turkey embryo, it became necessary to determine with considerable accuracy the extent of development of the embryo.

Phillips and Williams (1944) described the Black and the Beltsville Small White turkey embryos after different durations of incubation. However, chronological age, *i.e.*, incubation time, *per se*, is not a reliable expression of the extent of morphological differentiation of the embryo. Such factors as temperature and humidity during incubation, genetic composition, and size of the egg, have been shown to affect the rate of growth of the avian embryo (for review, see Landauer, 1951). It has previously been shown in this laboratory (Kosin and St. Pierre, 1956) that storage of Broad Breasted Bronze hatching eggs for 8 to 14 days results in a lowered mean somite count after 60 hours of incubation, as compared with eggs held for 1 to 7 days.

Hamburger and Hamilton (1951) established a series of normal stages of development for the chick embryo, based on various morphological criteria. These criteria were found to be useful in this laboratory in estimating the extent of development of the turkey embryo, although the turkey embryo takes approximately 28 days to hatch as compared with 21 days in the chicken. Thus, the major objective of the study reported in this paper was to determine for turkey embryos the period of incubation necessary to obtain the different stages of embryonic development described for the chicken by Hamburger and Hamilton (1951).

Similar studies on "staging" of embryonic development in Aves have previously been reported by Rempel and Eastlick (1957) and Koecke (1958) for the White Silkie bantam chicken and the Khaki Campbell and White Indian Runner ducks, respectively.

MATERIALS AND METHODS

All embryos used in this study were obtained from eggs produced by a flock of Broad Breasted Bronze (BBB) turkeys maintained at the Station. The birds

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[2] Supported, in part, by Federal Funds for Regional Research (W-7) under the Hatch Amended Act.

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[4] We wish to acknowledge the advice and help of Dr. Thomas J. Russell, Washington Agricultural Experiment Stations Statistician, in the analysis of the data on which this study is based. We are also indebted to Mrs. Lynne Frutiger, Mrs. Jewell Keeney, Mrs. Mary Ellen Sehy, and Mrs. Jeannette Wright for their technical assistance in the collection of these data.

were trapnested and the eggs were collected three times a day, after which they were placed in the holding room at 50° F. and 85% relative humidity for not more than three to four days. The eggs were incubated for a desired length of time in a forced draft incubator at 99.5° F. The earlier embryos (1 to 7 days) were removed from the yolk, placed in chick Ringer's solution and then measured and staged. Each embryo was staged separately according to (1) the development of the mesodermal derivatives, *e.g.*, somites; (2) the development of the ectodermal derivatives, *e.g.*, optic vesicles, neuromeres; and (3) the development of the heart. The "average" stage of development of the embryo was then obtained from these three separate stagings. Although there were individual differences, no striking and consistent differences between the turkey and the chicken were observed in terms of rate of development in these three groups of morphological criteria. Older embryos (17 to 28 days) were fixed in Bouin's fluid or Baker's calcium formol before staging.

In the later stages of development (Hamburger-Hamilton, stage 36 to stage 40), the turkey embryo has a distinct structure, the "snood" or "leader," which was included among the criteria used for describing the stage of development of the embryo.

Measurements of the beak and toe, which are the main criteria for identifying chick embryos from stage 40 to 44, were obtained for the turkey embryo. However, owing to the relatively small increments of increase in length in these structures, measurements of the foot, *i.e.*, from the outer edge of the tarsal joint to the tip of the claw of the third toe, were used to characterize the development from seventeenth to the twenty-seventh day of incubation.

This study is based on the observation of more than 4000 embryos collected over a period of three years.

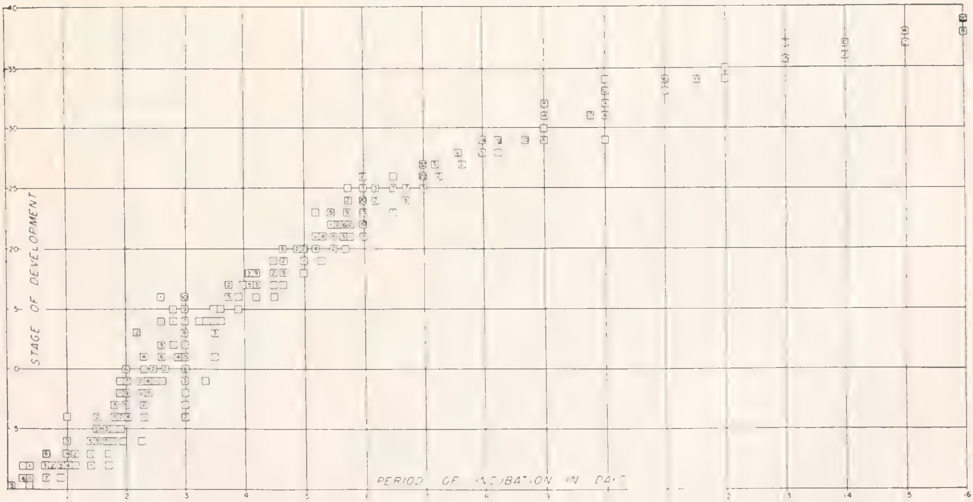


FIGURE 1. Stage (Hamburger and Hamilton, 1951) of development of the turkey embryo after various periods of incubation. The figures in boxes indicate the number of specimens for each point on the coordinate.

TABLE I

Anteroposterior (AP) lengths of the area pellucida and of the BBB turkey embryo at different stages of development

Stage	No. cases	AP length (mm.)	Sd	Embryo length (mm.)	Sd
4	11	3.3	.375	—	—
5	132	3.9	.423	—	—
6	233	4.3	.400	1.4*	.390
7	101	4.7	.458	2.3*	.242
8	52	5.3	.454	3.1**	.341
9	2	5.6		3.8	—
10	5	6.3	—	4.6	—
11	9	7.0	.508	5.6	.225
12	12	7.0	.673	5.8	.310

* From the head fold to Hensen's node.
** From the tip of the head to Hensen's node.

RESULTS AND DISCUSSION

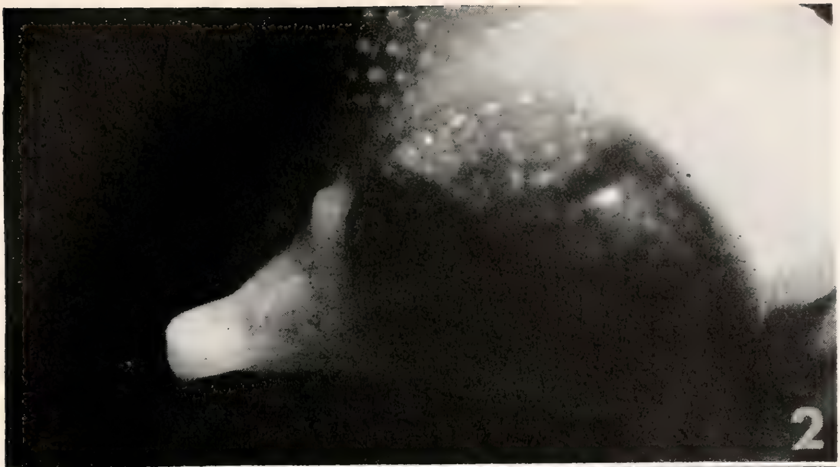
The approximate periods of incubation to obtain stages 1 to 39 can be obtained from Figure 1. There is a wide range of variation in the stage of development in the turkey embryo after a definite period of incubation. This becomes particularly apparent in the early stages.

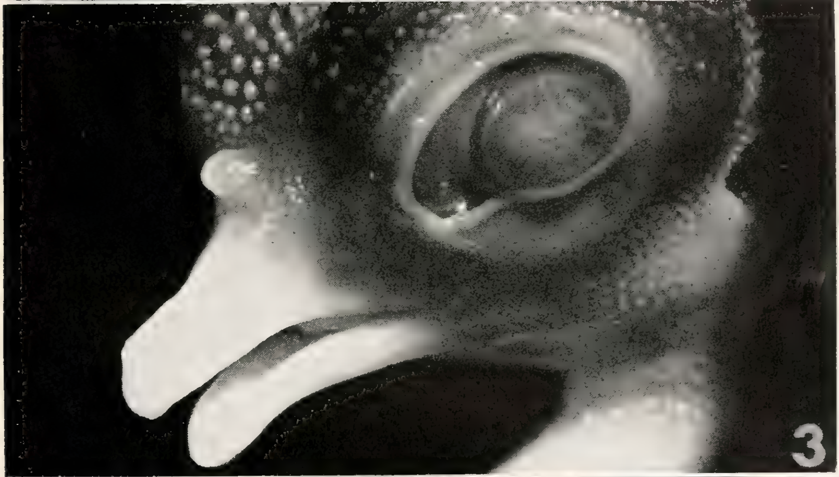
In the earlier stages (stages 4 to 12) the stage of the embryo can also be estimated from measurements of the anteroposterior lengths of the area pellucida or of the embryo, *i.e.*, from the tip of the head, or in stage 6, from the head fold to Henson's node (Table I). These measurements are highly correlated with the stage and somite number (Mun and Kosin, 1958).

The development of the snood ("leader") is summarized in the following tabulation:

Day of incubation	Hamburger & Hamilton Stage	Snood characteristics
13th day	36	Snood appears (Fig. 2).
12 to 14	37	Snood is as high as it is wide at the base (Fig. 3).
15	38	Snood is higher than wide and distinctly columnar in appearance (Fig. 4).
16 to 17	39	Snood is columnar and almost twice as tall as it is wide. Papillae may be seen at the base of the snood (Fig. 5).
17	40	The snood is covered with papillae (Fig. 6).
18	-	The snood is larger and conical feather germs at base of snood are colored (Fig. 7).
19th day	—	The feather germs are as long as the snood and may cover it completely (Fig. 8).

The growth of the beak, toe, and foot from the seventeenth to the twenty-seventh day of incubation is presented, graphically, in Figure 9. Each point represents the average measurements of 10 to 21 embryos by three different individuals on





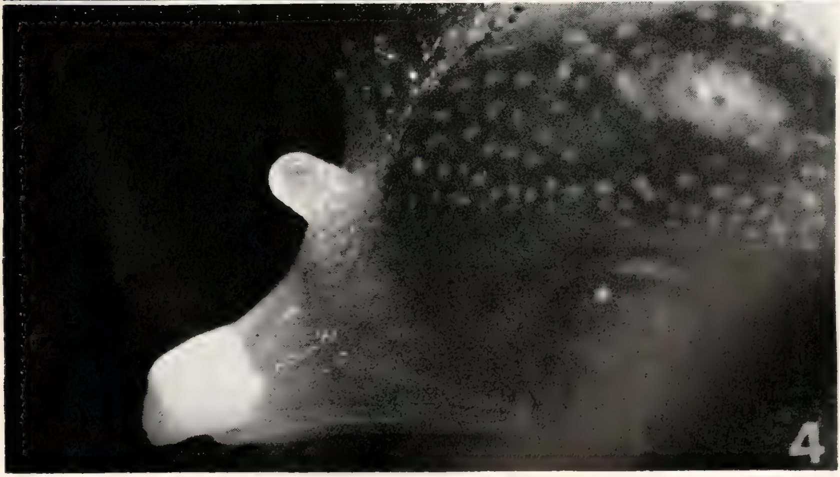
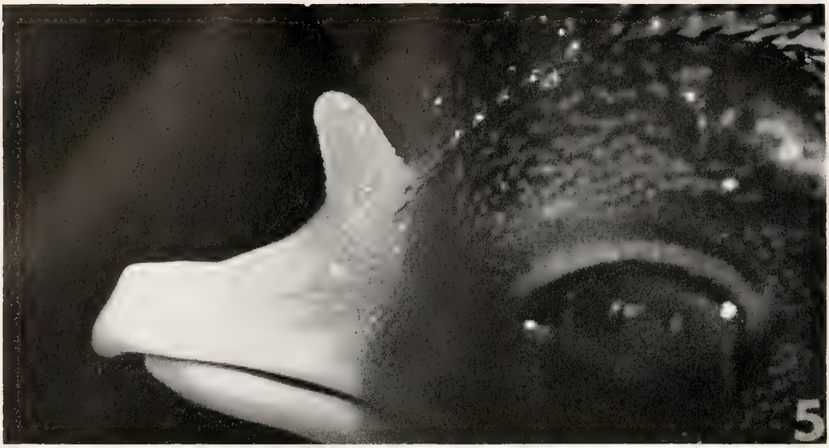
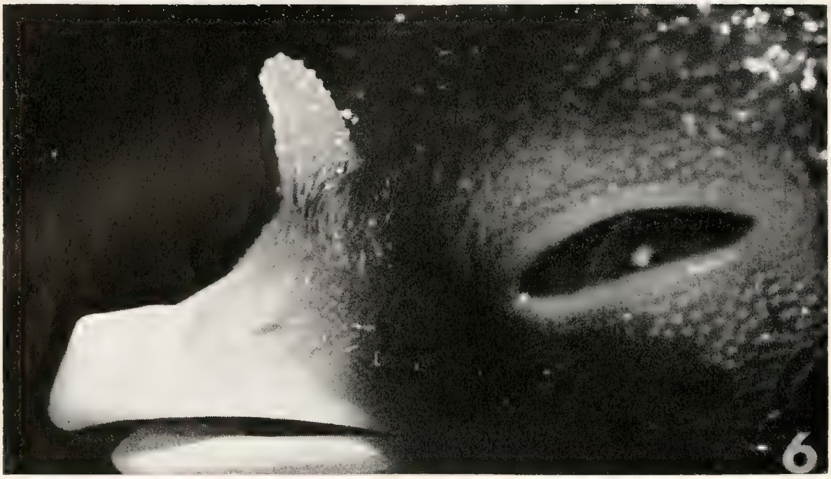


Figure 2. Snood at 13th day of incubation.
Figure 3. Snood at 14th day of incubation.
Figure 4. Snood at 15th day of incubation.





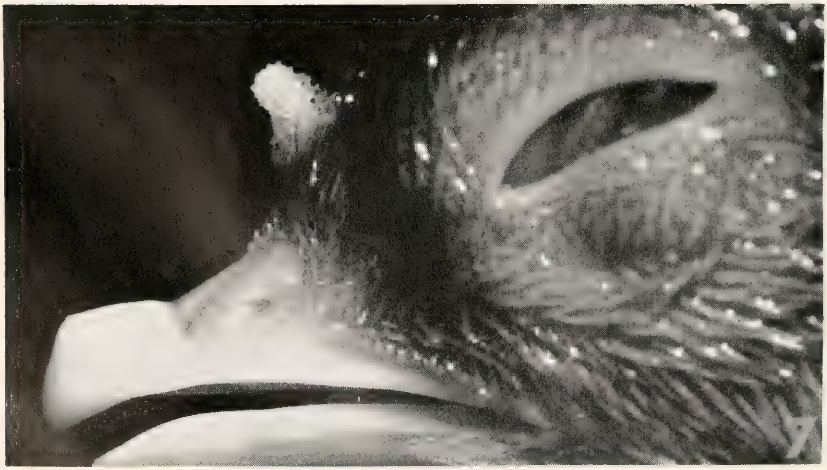


Figure 5. Snood at 16th day of incubation.
Figure 6. Snood at 17th day of incubation.
Figure 7. Snood at 18th day of incubation.

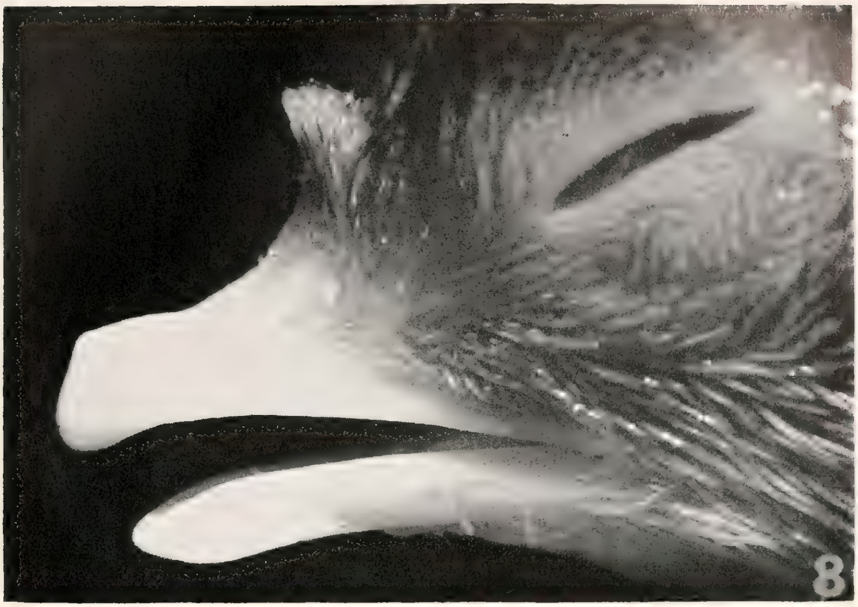


FIGURE 8. Snood at 19th day of incubation.

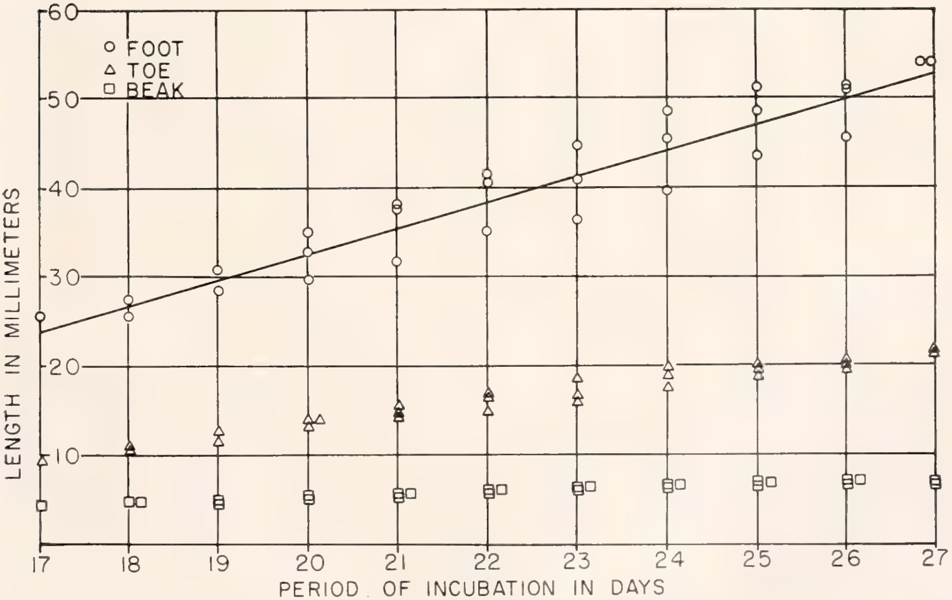


FIGURE 9. Length of beak, toe, and foot after different periods of incubation.

different groups of embryos at different times. The measurements were made with a pair of vernier calipers.

As may be seen from Figure 9, the rate of growth of the foot is greater than the rate of growth of the beak and toe. A straight line obtained from the regression of the length of the foot on time was then constructed. The equation of this line (solid line in Figure 9) is as follows:

$$Y = -25.3 + 2.9x$$

where Y = length of the foot in mm. and x = period of incubation in days.

The standard deviation of the regression line is 3.3 and the standard error of the slope is 0.051.

The 95% confidence interval in age of the embryo, for any particular length of the foot, can be calculated from the following equation:

$$\left.\begin{matrix}\bar{L}x\\ Lx\end{matrix}\right\} = 36.6 + 0.35Y_0 \pm 0.7\sqrt{0.00024(Y_0 - 40.0)^2 + 8.342}$$

where $\bar{L}x$ = upper limit of age of the embryo in days, Lx = lower limit of age of the embryo in days and Y_0 = observed length of the foot in millimeters.

Similarly, the 95% confidence interval for the length of the foot following a definite interval of incubation can be calculated from the following equation:

$$\left.\begin{matrix}\bar{L}y\\ Ly\end{matrix}\right\} = -25.3 + 2.9X_0 \pm 6.47\sqrt{1.002 + 0.00024(X_0 - 22.6)^2}$$

where $\bar{L}y$ = upper limit of the length in mm., Ly = lower limit of the length in mm. and X_0 = observed period of incubation in days.

This information has been used in our laboratory to approximate the time of death of the embryo, whether it was accidental, *e.g.*, due to incubation failures, or due to causes associated with the problem of hatchability, and to compare the growth rates of embryos from different lines of BBB turkeys cultivated *in vitro*.

SUMMARY

1. The period of incubation of Broad Breasted Bronze turkey eggs necessary to obtain the various normal stages of development established by Hamburger and Hamilton for the chick embryo is presented.

2. Data have been submitted on the development of the snood ("leader") in the turkey embryo.

3. Measurements of the beak, toe, and foot were obtained from the seventeenth to the twenty-seventh day of incubation. From these measurements, a straight line obtained from the regression of the length of the foot on time was constructed. The equation of this line is presented, as well as equations for determining the approximate age of the embryo from measurements of the foot, or the approximate length of the foot.

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CAROTENOIDS AND CHLOROPHYLLIC PIGMENTS IN THE MARINE SNAIL, CERITHIDEA CALIFORNICA HALDEMAN, INTERMEDIATE HOST FOR SEVERAL AVIAN TREMATODES[1, 2]

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The marine snail, *Cerithidea californica* Haldeman, is a favorable host for more than twenty species of larval trematodes (Martin, 1955; Hunter, 1942). These larvae occupy different regions of the body of the snail such as the digestive gland, mantle, gills and part of the digestive tract. The digestive gland of the snail, which is the main organ of infection, presents a variety of coloration in different specimens. It may be green, brown, yellow, orange or creamy white. The visceral part including the digestive tract is frequently dark blue. The mantle and the integument are usually greenish-blue or blue and yellow intermingled. A striking similarity exists between the coloration of the snail tissues and that of the parasitic larvae harbored by them.

A considerable amount of information is available concerning the occurrence and distribution of pigments, particularly carotenoids in various species of gastropod molluscs. Earlier work has been reviewed by Fox (1953) and Goodwin (1954). Although several species of snails are known to be hosts for pigmented larval trematodes, no critical study so far has been made concerning their pigments with a view to understanding the host-parasite relationship of pigmentation. In the snail, *Littorina littorea,* pigmented foot has been reported to be a means of recognizing infection with larval trematodes (Willey and Gross, 1957). Spectrophotometric absorption studies of *L. littorea* extracts indicated the presence of carotenoids; however, chromatographic methods were not used for the separation of various pigments. The author was interested to study the chemical nature and origin of pigments found in certain species of larval trematodes harbored by the snail, *Cerithidea* (Nadakal, 1960a, 1960b). In order to trace the host-parasite relationship of pigments, it was necessary to analyze the pigments of the snail. The present paper describes the pigments found in *Cerithidea* with special reference to carotenoids.

MATERIALS AND METHODS

Specimens of *Cerithidea* and four species of algae, including three green and one red algae which serve as food for the snails, were collected from the mud flats of Newport Bay, California. The green algae were identified as *Ulva* sp., *Chaeto-*

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morpha torta, and *Enteromorpha clathrata;* the red alga as *Hypnea johnstonii.* The algal pigments were studied by the methods of the following workers: Strain (1942) for chlorophyll *a* and *b;* Manning and Strain (1943) for chlorophyll *d;* and Haxo *et al.* (1955) for phycobilins. After sorting out the various snail tissues such as the digestive gland, mantle and foot, and visceral mass, they were lyophilized separately and ground in an ordinary mortar. The methods outlined by Fox and Pantin (1941) were followed with necessary modifications for the extraction and analysis of pigments from the snail tissues. Chromatographic separation of pigments was carried out according to the directions given by Karrer and Jucker (1950). A cylindrical glass tube measuring 20 cm. × 12 mm. was used for preparing the adsorption column. Among several adsorbents tried for the separation of various pigments, such as calcium hydroxide for epiphasic carotenoids, calcium carbonate and zinc carbonate for hypophasic carotenoids, and powdered sugar (C & H Confectioner's) for chlorophyll derivatives, activated alumina was found most satisfactory. Various pigment fractions obtained by chromatographic separation were eluted in appropriate solvents like petroleum-ether, methanol, etc. for determining the absorption spectra, with a Beckman Spectrophotometer. Efforts were made to identify the pigments by spectrophotometric absorption analyses, partition tests, color reactions, solubility, fluorescence, and chromatographic behavior.

Figures 1–3 include spectral curves of the pigments A-E in petroleum-ether (b.p. 50–70° C.) and F and G in methanol.

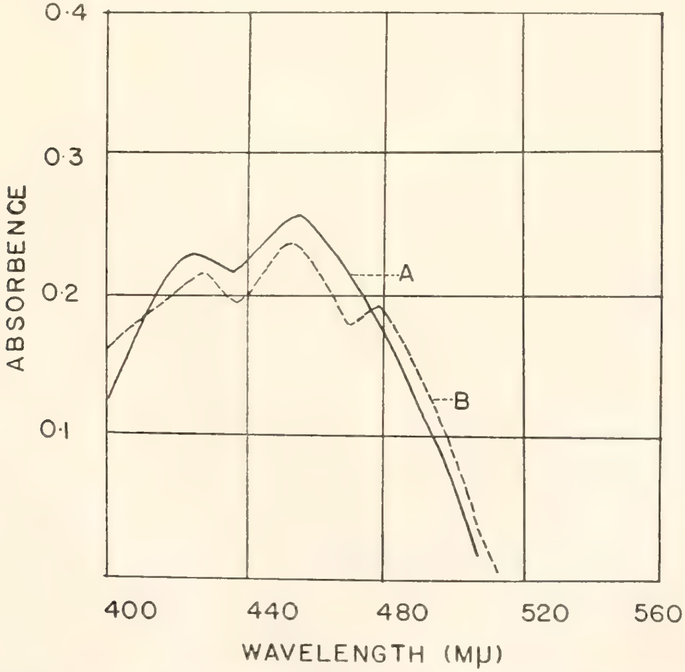

FIGURE 1. A. Light orange pigment HIII (Table II). B. Yellow pigment HII (Table II).

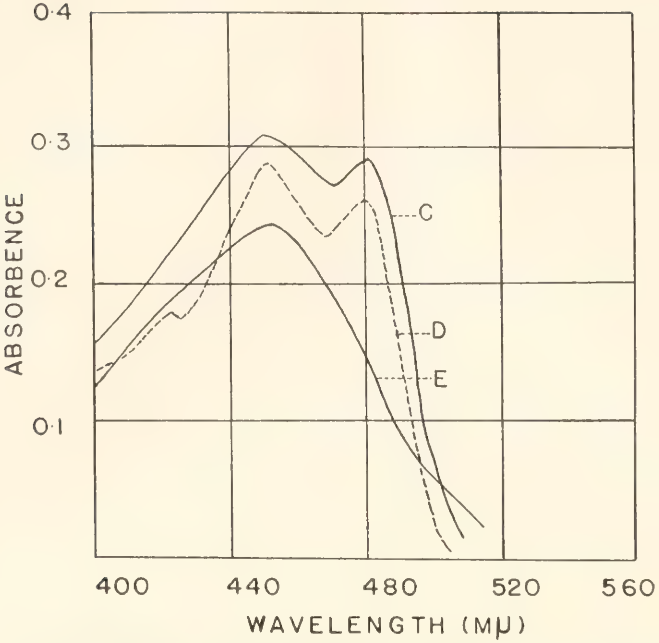

FIGURE 2. C. Orange pigment EIV (Table I). D. Orange pigment EIII (Table III). E. Violet pigment EIII (Table I).

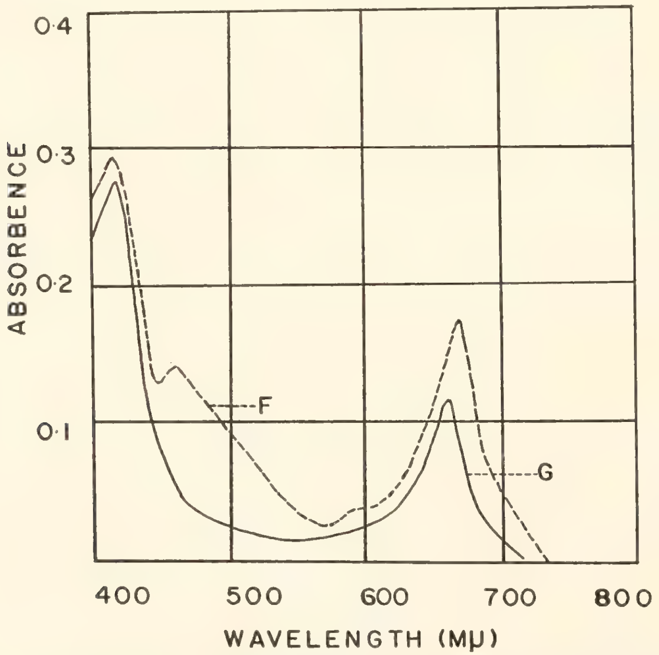

FIGURE 3. F. Yellowish-brown pigment EII (Table I). G. Pale green pigment EIIa (Table I).

RESULTS

The pigment fractions obtained by chromatographic separation of the epiphasic and hypophasic portions of the pigment extracts of the various snail tissues and their characteristics are listed in Tables I-V. The pigment fractions in the case of epiphasic portions of the extracts are numbered in order of decreasing adsorption and hypophasic portions in order of increasing adsorption on the columns.

Absorption maxima and the forms of the spectral curve (Fig. 3, G) of the pigments EI and EIIa (Table I) indicate that these pigments resemble chlorophyll or pigments derived from chlorophyll. However, the absorption maxima in the violet region of the spectrum are different from that of the chlorophyll reported from plant sources. The Gmelin reaction (Pearse, 1953) was negative for these pigments, suggesting that they are not open-ring tetrapyrrole compounds.

The brown pigment EIIb (Table I) showed a maximum at 450 mμ. This fraction could not be made hypophasic even after prolonged saponification. This may be a carotenoid acid.

TABLE I

Epiphasic portion of the digestive gland extract. Adsorbent: activated alumina. Developing solvent: petroleum ether (50–70° C.) with 1–5% methanol

Band No.	Color of band	Percentage of solvent required for elution	Absorption maxima mμ	Solvent
EI	Greenish-yellow	2–3 methanol	410, 667	Ethanol
EII	Yellowish-brown	2–3 methanol	410, 450, 667	Ethanol
EIII	Violet	3–4 methanol	454	Petroleum ether
EIV	Orange	0	452, 482	Petroleum ether

Band EII was chromatographed again on alumina column and the two fractions obtained are given below:

Band No.	Color of band	Percentage of solvent required for elution	Absorption maxima mμ	Solvent
EIIa	Pale green	2–3 methanol	416, 665	Methanol
EIIb	Brown	3–4 methanol with a few drops of glacial acetic acid	450	Petroleum ether

The violet pigment EIII (Table I) showed a maximum at 454 mμ. The form of the spectral curve (Fig. 2, E) and the single absorption maximum are suggestive of a keto-carotenoid (Vevers and Millott, 1957).

The orange pigment EIV (Table I) has been found to possess properties similar to those of β-carotene (Fig. 2, C). The absorption maxima are in good agreement with the figures given by Karrer and Jucker (1950), and Lederer (1938). Besides, solubility, behavior on partition test, fluorescence (bluish-green in ultra violet light), color reactions (pigment in chloroform solution turned bluish-green on addition of concentrated sulfuric acid), color in solutions, and chromatographic behavior also indicate that this is β-carotene.

The absorption maxima of the pink pigment HI (Table II) in petroleum ether and benzene are in good agreement with the figures given by Karrer and Jucker (1950), and Goodwin (1953) for zeaxanthin. Its hypophasic behavior on partition test and chromatographic behavior also lend support to the conclusion that

TABLE II

Hypophasic portion of the digestive gland extract. Adsorbent: activated alumina. Developing solvent: petroleum ether with 1–5% methanol

Band No.	Color of band	Percentage of solvent required for elution	Absorption maxima mμ	Solvent
HI	Pink	2–3 ethanol	420, 450, 482	Petroleum ether
			462, 490	Benzene
HII	Yellow	1–2 methanol	426, 448, 478	Petroleum ether
			429, 457, 487	Chloroform
HIII	Light orange	1–2 methanol with a few drops of glacial acetic acid	424, 454	Petroleum ether
HIV	Blue-green	2–3 ethanol	416, 670	Methanol

this is zeaxanthin. The fact that this pigment fraction stayed hypophasic even after continued saponification and could not be made epiphasic with acid treatment, showed that this pigment occurs in the snail tissues in the free state.

The yellow pigment HII (Table II) showed maxima in petroleum ether and chloroform which are in good agreement with the figures quoted by Karrer and Jucker (1950) for the xanthophyllic pigment lutein (Fig. 1, B). This pigment turned bluish-green with concentrated sulfuric acid. These properties, coupled with the chromatographic behavior, hypophasic nature on partition test, and color in solutions, prove that this is lutein.

A yellow pigment fraction, separated from the epiphasic portion of the unsaponified pigment extract, showed maxima in petroleum ether at 424, 445, and 475 mμ. This pigment became hypophasic on saponification and could be made epiphasic again with acetic acid treatment. This indicated that some of the lutein in the snail's tissues is esterified. After saponification the pigment showed the maxima at 446 and 478 mμ in petroleum ether.

The light orange pigment HIII (Table II) could be removed from the column with a few drops of acetic acid in the eluting solvent. It is difficult to identify this pigment conclusively; it may be a carotenoid acid or some pigment derived from hypophasic carotenoids (Fig. 1, A).

The blue-green pigment HIV (Table II) is characterized by absorption maxima and spectral curve suggestive of chlorophyll or a pigment derived from it.

The brown pigment EI (Table III) showed only one absorption maximum.

TABLE III

Pigment extracts from the mantle, branchial, and pedal tissues. No hypophasic fraction was obtained on partition of the original extracts between 90% methanol and petroleum ether systems. Pigments chromatographed as the epiphasic portion of the digestive gland extract

Band No.	Color of band	Percentage of solvent required for elution	Absorption maxima mμ	Solvent
EI	Brown	2–3 methanol	450	Petroleum ether
EII	Pink	1–2 methanol with a few drops of glacial acetic acid	452	Petroleum ether
EIII	Orange	0	424, 452, 480	Petroleum ether

This fraction could not be made hypophasic even after prolonged saponification in 90% methanol-petroleum ether systems. Identification of this pigment fraction was difficult.

The pink pigment EII (Table III) could be eluted with a few drops of glacial acetic acid. It showed a maximum at 452 mμ. On saponification this became hypophasic and could be made epiphasic again by treating with acetic acid. This behavior indicated that this pigment could be an esterified carotenoid acid.

The orange pigment EIII (Table III) showed all characteristics of β-carotene. However, a small shoulder at 424 mμ in the spectral curve of this pigment is remarkable (Fig. 2, D).

The khaki pigment EI (Table IV) showed an absorption maximum only in the violet region of the visible spectrum. This may be some breakdown product of chlorophyll or carotenoid pigments. Such breakdown products are known to be adsorbed at the top of the columns (Fox, 1953).

TABLE IV

Pigment extract from the visceral mass. Epiphasic portion chromatographed on activated alumina. Developing solvent: petroleum ether with 1–5% methanol

Band No.	Color of band	Percentage of solvent required for elution	Absorption maxima mμ	Solvent
EI	Khaki	1–2 methanol	425	Methanol
EII	Greenish-yellow	2–5 methanol	416, 667	Methanol
EIII	Yellowish-brown	2–4 methanol	420, 452, 667	Methanol
EIV	Violet	2–3 methanol	452	Petroleum ether
EV	Orange	0	450, 482	Petroleum ether

Band EIII was chromatographed again on alumina column and the two fractions obtained are given below:

Band No.	Color of band	Percentage of solvent required for elution	Absorption maxima mμ	Solvent
EIIIa	Pale green	1–2 methanol	416, 665	Methanol
EIIIb	Pale orange	1–2 methanol with a few drops of glacial acetic acid	450	Petroleum ether

The spectral properties of the pigments EII and EIIIa (Table IV) indicated that they are chlorophyll derivatives.

The pale orange pigment EIIIb (Table IV) is considered to be a carotenoid acid because of its single absorption band and acidic properties.

The violet pigment EIV (Table IV) was more or less similar to the violet pigment EIII (Table I) extracted from the digestive gland and may be a keto-carotenoid.

The orange pigment EV (Table IV) was identical with the pigments EIV (Table I) and EIII (Table III) extracted from the digestive gland and mantle, respectively. It is therefore concluded to be β-carotene.

The pink pigment HI (Table V) was similar in properties to the one HI (Table II) recovered from the hypophasic portion of the digestive gland extract and is concluded to be zeaxanthin.

The yellow pigment HII (Table V) has been identified as lutein, as its properties resemble those of the yellow pigment HII (Table II) extracted from the hypophasic portion of the digestive gland extract.

The light orange pigment HIII (Table V) with its acidic properties and two absorption maxima in the blue-violet region of the visible spectrum may be considered as a carotenoid acid. No trace of chlorophyll derivatives could be detected in the hypophasic portion of the visceral extracts.

A blue-green residue was left in the methanol-water fraction of the original extracts of the digestive gland and visceral tissues. Part of the colored substance could be taken up in ether after addition of a few drops of glacial acetic acid. It was then washed with water, evaporated to dryness under vacuum and finally taken up in methanol. The absorption maxima of this pigment at 416 and 667 mμ and the form of the spectral curve was characteristic of chlorophyll *a* (Atkins and Jenkins, 1953; Green, 1957). Even after extraction of the chlorophyllic pigments by acidified ether, a bluish residue was left behind in the aqueous methanolic solution. This was not extractable by any of the solvents tried. Attempts to separate the pigment on adsorption columns also failed. This bluish residue probably contained haemocyanin which is common in molluscan body fluids.

TABLE V

Hypophasic portion of the visceral extract. Chromatographed as the hypophasic portion of the digestive gland extract

Band No.	Color of band	Percentage of solvent required for elution	Absorption maxima mμ	Solvent
HI	Pink	2–3 methanol	420, 450, 482	Petroleum ether
HII	Yellow	1–2 methanol	422, 448, 478	Petroleum ether
HIII	Light orange	1–3 methanol with a few drops of glacial acetic acid	424, 454	Petroleum ether

PIGMENTS FOUND IN THE ALGAE

The three species of green algae, *Ulva* sp., *Enteromorpha clathrata*, and *Chaetomorpha torta*, have been found to contain chlorophyll *a* and *b*, β-carotene, and the xanthophyllic pigment lutein. No trace of α-carotene or any other pigments related to carotenoids could be detected. The red alga, *Hypnea johnstonii*, contained chlorophyll *a* and *d*, β-carotene, lutein, phycoerythrin, and phycocyanin. The principal carotenoid pigment extracted from the algae was β-carotene.

DISCUSSION

The occurrence of carotenoids and chlorophyll derivatives in *Cerithidea* is in agreement with the previous findings in various species of molluscs. β-Carotene and lutein have been reported from several gastropods (Fox, 1953; Goodwin, 1954). Zeaxanthin is known to occur in *Patella vulgata* and *P. depressa* (Goodwin, 1954; Goodwin and Taha, 1950), and *Mytilus californianus* (Scheer, 1940). Carotenoid acids with single absorption bands in the visible spectrum have also been reported from many invertebrates such as sponges and molluscs (Fox, 1953). Demonstration of chlorophyll derivatives in molluscs has been made by MacMunn (1886a, 1886b), Dhéré and Vegezzi (1916), and many others.

A survey of the occurrence and distribution of carotenoid pigments in invertebrates reveals the fact that the digestive gland plays an important role in the storage of these pigments. Crane (1949) found that in the cephalopods, *Octopus bimaculatus* and *Loligo opalescens*, the liver-pancreas accumulated relatively large amounts of carotenoid pigments. In *Cerithidea*, undoubtedly the digestive gland functions as the chief organ for the storage of carotenoids. The preferential accumulation of carotenoids in the lipid-rich digestive gland is not surprising since these pigments have a tendency to be associated with lipids.

The visceral extracts of *Cerithidea* have also been found rich in carotenoid and other pigments. This might be due to the presence in the gut of plant materials ingested as food. The mantle and branchial tissues contained relatively small amounts of carotenoids and no traces of chlorophyll derivatives could be detected in these tissues. A similar situation is described by Brooks and Paulais (1939) in the lamellibranchs, *Ostrea edulis* and *Gryphaea angulata*.

In *Cerithidea*, the principal pigment found in various tissues is β-carotene. Next in importance, on a quantitative basis, are the chlorophyll derivatives; third comes the lutein and fourth only the zeaxanthin. Carotenoid acids and keto-carotenoids occur in traces only. The snail apparently shows a preference for storing β-carotene in its tissues. This is perhaps due to the preponderance of β-carotene in the algae which serve as food for the snail. Since *Cerithidea* assimilates and stores both hydrocarbons and xanthophylls as well as chlorophyll derivatives, it may be regarded as non-selective in its chromatic storage. However, the preferential accumulation of carotenes in the mantle and the branchial tissues is remarkable indeed.

There is ample evidence for the elementary origin of carotenoids and chlorophyll derivatives in animals. The crustacean, *Daphnia*, builds up its carotenoid supply from the algae upon which it feeds (Green, 1957). The sea mussel, *Mytilus californianus*, is known to absorb and store carotenoid pigments "from a very plentiful and widely varied diet" (Fox and Coe, 1943). Dhéré and Vegazzi (1916) concluded from experimental evidence that the greenish and grayish hepatic pigments of *Helix pomatia* were derived from the chlorophyll of its diet. The dark green pigment "chaetopterin" found in the intestinal epithelium of the polychaete worm, *Chaetopterus*, is derived from chlorophyll by the elimination of magnesium and the phytol chain (Lederer, 1940). Similarly, Lederer and Huttrer (1942) and Winkler (1957) showed that the sea slug, *Aplysia*, accumulates the pigment "aplysioviolin" in its ink-gland, which is derived from red algae consumed as part of its diet.

As regards *Cerithidea*, sources of pigments could also be attributed to nutritional factors. Examination of four species of algae which serve as food for the snail indicated the presence of chlorophylls and carotenoids, in addition to the phycobilins and chlorophyll *d* in the red alga. The snail probably builds up its pigment supply from these algal sources. β-Carotene seems to have been accumulated in the snail tissues without any metabolic alteration. However, the bluish-green color of mantle tissues is an indication that carotenoids may exist in them as a carotenoid-protein complex. The fact that no chlorophyll derivatives could be detected from these tissues also lends support to this conclusion. Zeaxanthin, carotenoid acids, and keto-carotenoids were not observed in the algae studied; they may be considered as products of metabolic activities of the snail. Moreover, some of the lutein and carotenoid acids were found esterified in the tissues of the snail. The spectral properties of chlorophyll derivatives indicate that these pigments must have also undergone some kind of metabolic change, possibly oxidation. The absorption maxima of the chlorophyll derivatives in the red region of the spectrum suggest that these pigments are derived from chlorophyll *a* of the algae. There was no indication of the presence of chlorophyll *d* or phycobilins in the tissues of the snail, negating the possibility that the snail absorbs these pigments from the red alga, *Hypnea*.

Several examples can be cited to prove that the accumulation of pigments in the body tissues of animals frequently results from catabolic activities. Such accumulation of pigments may or may not be significant in the functional economy of these organisms. It has been reported that the large pigment cells found in the deeper layer of connective tissues adjoining the intestinal caeca of the leech, *Glossiphonia complanata,* represent a kidney for the storage of waste products derived from haemoglobin metabolism (Bradbury, 1957). Wigglesworth (1943) found that in the blood-sucking bug, *Rhodnius prolixus,* some of the ingested blood is denatured to form biliverdin which is subsequently either excreted through the gut or stored in the pericardial cells. Stephenson (1947) noticed that the pigment in the gut epithelium of *Fasciola hepatica* is derived from the haemolysis of the ingested blood. The occurrence of chlorophyll derivatives in *Cerithidea* may not have any functional significance; they simply happen to be deposited in the tissues as metabolic wastes resulting from the digestion of algal food. Nevertheless, the storage of carotenoids in various tissues, particularly in the digestive gland of the snail, may be beneficial since there are indications that certain carotenoids may serve to prevent autoxidation of lipids in animal tissues (Verne, 1936a, 1936b). It is yet to be found out whether the snail needs vitamin A for its metabolic activities, and, in case it does, it might make use of β-carotene as a potential source. Although in certain invertebrates carotenoids are known to be utilized in sexual reproduction (Scheer, 1940) and in maintenance of mucous surfaces, nothing is known about their roles in similar processes in *Cerithidea*.

SUMMARY

1. Evidences obtained from chromatography, spectrophotometric absorption analyses, partition tests, etc. suggested the occurrence of the following pigments in the marine snail, *Cerithidea californica*: β-carotene, carotenoid acids, keto-carotenoids, lutein, and chlorophyll derivatives.

2. In an attempt to understand the dietary relationship of pigmentation in the snail, four species of algae were studied for their pigment contents. The three green algae were found to contain chlorophyll *a* and *b*, β-carotene, and lutein; the red alga, chlorophyll *a* and *d*, β-carotene, lutein, and phycobilins.

3. The spectral properties of the chlorophyll derivatives recovered from the snail suggested that they are derived from chlorophyll *a* of the algae and that the molecular structure is still intact with the magnesium atom atached to it. However,

absorption maxima in the violet region of the spectrum are shifted toward shorter, and in the red toward longer wave lengths, indicating some metabolic change in these pigments, possibly oxidation.

4. Part of the lutein and carotenoid acids were found to be esterified in the digestive gland and mantle tissues. No metabolic alteration has been noticed in the case of β-carotene. All available evidence suggests that zeaxanthin, carotenoid acids, and keto-carotenoids are products of the snail's metabolic activities.

5. Apparently the snails do not absorb phycobilins or chlorophyll *d* from the red alga.

6. The snail has been found to be non-selective in its chromatic storage.

7. The nutritional relationship and biological significance of pigments in the snail have been discussed.

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THYROID HORMONE TREATMENT AND OXYGEN CONSUMPTION IN EMBRYOS OF THE SPINY DOGFISH [1]

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The inability of adult cold-blooded vertebrates to respond to thyroxine treatment with an increased oxygen consumption rate is now a well documented finding (Hoar, 1957; Gorbman, 1959). The two often cited exceptions to this general experience are the thyroxine-induced increases in oxygen consumption in adult goldfish observed by Müller (1953), and in parrot fish of certain sizes, as described by Smith and Matthews (1948). Both of these claims have been denied by opposite results in the same species (Etkin, Root and Mofshin, 1940; Chavin and Rossmore, 1956; Matty, 1957). Measurements of metabolic rate in fishes are subject to numerous variables which are not as easily controlled as they are in mammals (responses to handling, previous temperature history, illumination, endogenous activity cycles) (Fry, 1957), so that it is not surprising that conflicting claims may exist for some species. Of the factors which may contribute misleading information in measurements of oxygen consumption in fishes, among the most significant is muscular activity. Hoar (1958) has shown clearly that treatment of fishes with thyroid hormone induces behavioral changes, expressed primarily by an increased spontaneous motor activity. If this is so, then any valid test for basal metabolic stimulation by thyroxine must exclude the variable of locomotor muscular work. Although testing systems are available which make this possible (Fry, 1957), neither of the two exceptional claims mentioned above utilized them.

While working with near-term embryos of the spiny dogfish, *Squalus suckleyi*, removed from the uterus and kept in flowing sea water, we noticed a behavioral feature which makes this animal useful in respiratory studies. When kept in subdued light they remain still, even after treatment with thyroid hormone. Since under these circumstances, spontaneous muscular movements are rare, then respiratory measurements can be taken to reflect "basal" requirements (or at least "standard" metabolism as defined by Fry, 1957), not a thyroxine-induced increase in swimming. In these experiments oxygen consumption of such exteriorized dogfish pups was measured after treatment with thyroxine, or two of its analogues, or propyl thiouracil. To our knowledge the only other studies of the metabolic responsiveness to thyroid hormone in larval vertebrates have concerned anuran tadpoles. In this regard, too, the published literature is in disagreement (Etkin, 1955; Lewis and Frieden, 1959). Accordingly, it was hoped that the experiments with dogfish embryos would prove enlightening, both with regard to the metabolic action of thyroid hormone in poikilotherms and its action in differentiating forms of such animals.

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Material and Methods

Oxygen consumption was measured in a continuous-flow apparatus of the type used by Job (1955) in measuring the "standard" metabolism of trout. Four respirometer flasks (2.5-liter Fernbach culture jars) were used at any one time, one of these being used as a "blank." The four flasks were immersed in a large wooden tank in which the water level was maintained constant by means of an overflow. The flow of water through the flasks was so adjusted that *Squalus* embryos in groups of three removed about 0.5 to 1.0 ml. of oxygen. Rate of oxygen consumption was calculated from the flow rate, the difference in oxygen content between incurrent and excurrent water, and the wet weight of the animals tested. Oxygen content of the water was determined by the unmodified Winkler technique.

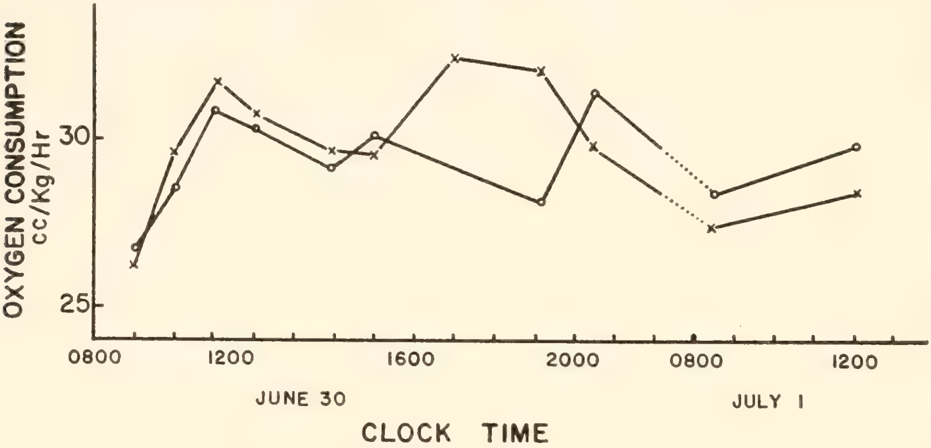

Figure 1. Serial determination of metabolic rate in untreated embryos of *Squalus suckleyi* over a 27-hour period. Two respirometers used with three pups in each.

Water temperature never varied more than 1° C. during a single series of tests, and usually it did not change at all measurably. During most measurements of oxygen consumption the water temperature ranged from 13° to 14° C. However, extremes of 12° C. and 16° C. (on one unusually hot day) were recorded. The water in the large water bath was continuously aerated through a stone "air breaker," and preliminary tests showed that the oxygen content of the water was uniform at all points in the bath.

Embryos were tested in groups of three, being placed in the respirometers one hour before the first measurement of oxygen consumption. The bath containing the respirometers was covered to shield the animals from most of the light and other extraneous factors. Frequent observation indicated that under these conditions spontaneous muscular movements were rare. Several prelimi-

TABLE I

Metabolic rates of non-treated dogfish pups showing normal day-to-day variability in the laboratory. All runs made between 1:00 and 3:00 PM

Date	No. of trials	Mean O_2 consumption (cc./kg./hr.)
July 3	4	28.8 ± 1.4 (std. dev.)
July 4	4	31.4 ± 1.9
July 6	3	29.5 ± .89
July 8	3	31.3 ± 1.4
July 10	3	33.7 ± 1.7

nary measurements were made on untreated pups, to ascertain the variability in oxygen consumption under laboratory conditions. Figure 1 illustrates the results of serial determinations on two groups of embryos, all taken from the same female, over a $1\frac{1}{2}$-day period. The period of steadiest metabolic rate was in the afternoon; accordingly all measurements reported here were made in the afternoon only. In another preliminary test, metabolic rates were determined on pups from one female dogfish, over a period of one week immediately following transfer of the pups to the laboratory. The results (Table I) show that the average metabolic rate remained at about the same level over this period and that the variability in oxygen uptake (as indicated by the standard deviations) was quite small.

TABLE II

Experimental protocol for each injection group. Numbers in parentheses indicate the number of pups from that female

Experiment	Total no. of injections	Females from which pups were taken	Substances tested*	Dose per injection
1	10	A(15)	triac l-Tx	10 μg. 10 μg.
2	9	B(11), C(9)	triac l-Tx PTU	10 μg. 10 μg. 50 μg.
3	9	D(10), E(10)	triac l-Tx PTU	10 μg. 100 μg. 10 μg. 50 μg.
4	8	F(11), G(4), H(4), I(4), J(5), K(7)	triac l-Tx T3 PTU	10 μg. 100 μg. 10 μg. 100 μg. 50 μg.
5	5	L(19)	l-Tx T3 PTU	100 μg. 100 μg. 50 μg.
6	4	M(25), N(10)	triac l-Tx	10 μg. 10 μg.

* Abbreviations: triac, triiodothyroacetic acid; l-Tx, l-thyroxine; PTU, propylthiouracil; T3, triiodothyronine.

Table III

Effects of repeated injections of thyroid hormones on oxygen consumption (cc./kg./hr.) of embryos of Squalus suckleyi.* *Values in the table are means of 3 to 6 consecutive determinations, taken at 15-minute intervals. E = experimental. C = control*

Experiment	No. of injections		Triac 10 µg.	Triac 100 µg.	l-Tx 10 µg.	l-Tx 100 µg.	T3 100 µg.	PTU 50 µg.
1	1	E	32.40		33.52			
		C	30.79		32.52			
		E/C × 100	105		103			
	4	E	52.25		44.78			
		C	39.28		39.28			
		E/C × 100	133		114			
	9	E			46.82			
		C			44.60			
		E/C × 100			105			
2	2	E	32.25		32.15			28.78
		C	31.52		31.52			31.52
		E/C × 100	102		102			91
	4	E	41.90		40.90			36.00
		C	35.80		35.80			35.80
		E/C × 100	117		114			101
	9	E	33.80		41.40			31.27
		C	34.16		34.16			34.16
		E/C × 100	99		121			91
3	1	E	33.10	33.40	33.30			32.70
		C	33.20	33.20	33.20			33.20
		E/C × 100	99	101	100			98
	4	E	36.70	33.90	29.20			29.00
		C	30.40	30.40	30.40			30.40
		E/C × 100	121	108	96			98
	9	E	35.87	35.50	33.62			38.09
		C	32.96	32.96	32.96			32.96
		E/C × 100	109	108	102			115
4	2	E	34.13	32.56	30.82		30.12	30.30
		C	29.86	29.86	29.86		29.86	29.86
		E/C × 100	114	109	103		101	102
	5	E	37.02	33.45	33.97		34.63	32.51
		C	30.41	30.41	30.41		30.41	30.41
		E/C × 100	122	110	112		114	107
	8	E	32.33	33.66	31.86		32.28	29.44
		C	27.49	27.49	27.49		27.49	27.49
		E/C × 100	118	122	116		118	107

* This table includes absolute values for oxygen consumption at the beginning (after 1 or 2 injections), the middle (after 4 or 5 injections), and end after 8 or 9 injections in each experiment. The complete course of each experiment is shown in Figures 4A, 4B and 4C, but these do not show absolute values. To tabulate all the absolute values would require an impractically long table.

TABLE III—*Continued*

Experiment	No. of injections		Triac 10 µg.	Triac 100 µg.	l-Tx 10 µg.	l-Tx 100 µg.	T3 100 µg.	PTU 50 µg.
5	1	E				30.01	25.78	26.96
		C				26.86	22.82	22.82
		E/C × 100				112	113	118
	5	E				39.92	42.96	37.07
		C				35.78	35.78	35.78
		E/C × 100				112	120	104
6	1	E	28.49		30.37			
		C	26.18		26.18			
		E/C × 100	109		110			
	4	E	35.34		32.82			
		C	29.78		29.78			
		E/C × 100	119		110			

The animals used were "pups" removed from the uteri of *Squalus suckleyi* females caught during July and August, 1958, at Friday Harbor, Washington, within 200 yards of the laboratory. The ovoviviparous young of this species remain in the uterus for two years. It could be estimated from the sizes of the yolk sacs that the embryos we used were approximately 19–23 months of age. Occasional "spontaneous" birth of pups of captive females was observed in late August. Embryos removed from the uterus were kept in apparently good condition for periods as long as several weeks in large, covered glass dishes (2-liter capacity) in groups of 4 or 5, in slowly flowing sea water. Whenever possible, all pups used in an experiment were taken from the same mother. In experiments requiring large numbers of pups it was necessary to combine litters from several mothers and these were distributed as evenly as possible into the different experimental groups (Table II). The limited number of embryos of equivalent development available at one time, and the limited capacity of the respirometers made it impossible to test all hormones at the same time. For this reason, six different experiments were performed during an eight-week period.

In each experiment groups of embryos were injected intraperitoneally on alternate days with various doses (Table II) of hormones, propylthiouracil, or 0.7% NaCl solution, always in a volume of 0.05 cc. Oxygen consumption was determined on the day after injection to avoid possible responses to handling.

The compounds tested for their effect on oxygen consumption were l-thyroxine (Tx), l-triiodothyronine (3:5:3′-triiodo-l-thyronine, T$_3$), triiodothyroacetic acid (3:5:3′ triiodothyroacetic acid, Triac) in doses of 10 micrograms or 100 micrograms, and propylthiouracil in doses of 50 micrograms.

EXPERIMENTS AND RESULTS

Six experiments (Table III) were completed. The total number of injections, given at 2-day intervals, was as few as 4 or 5, but was usually (in four of the six experiments) 8 to 10. The shorter experiments were ended when accidental blockage of the sea water occurred. Since such occurrences were obviously harmful, and their effects difficult to assay, respiratory measurements were accordingly not continued. In experiment 1 such a blockage killed all Triac-injected animals after the fifth injection (Fig. 2). The Tx-injected animals in this experiment showed an extreme but temporary respiratory depression (Fig. 2) at the same time, so may have had a brief experience of the same nature. Only one other extremely variant datum is seen in Figure 3, which shows the results of experiment 2. Here an exceptionally high respiratory rate was observed in saline solution-injected controls after three injections. Since these same animals in succeeding measurements showed relatively little variation in oxygen consumption, it is felt that the exceptional figure may have been due to some limited experience, possibly a brief interruption in water supply. The remaining data, summarized in the figures, and Table III, appear to show consistent trends of response, or lack of response, to the various forms of treatment.

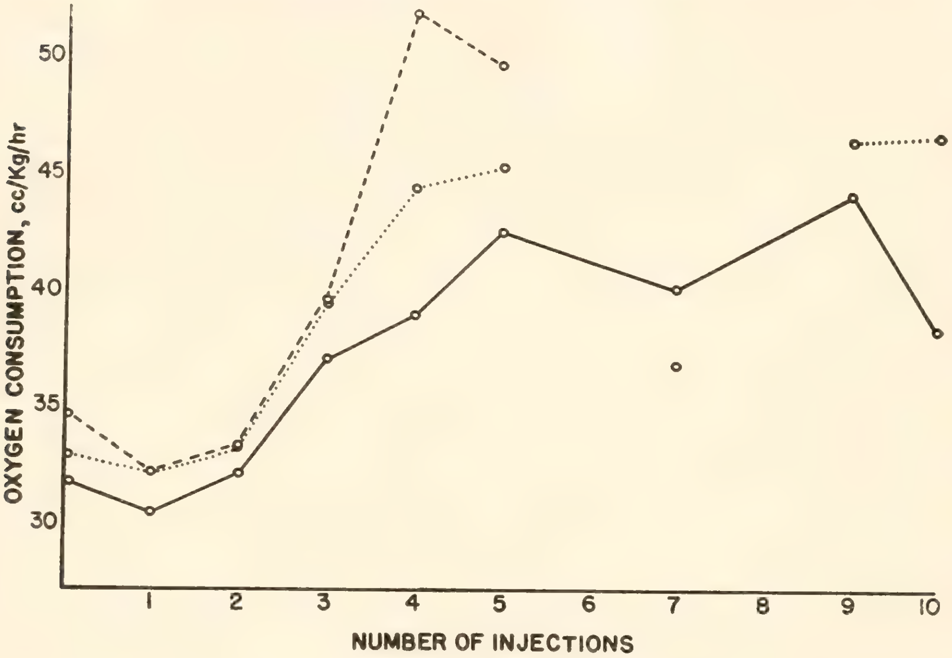

FIGURE 2. The effects of repeated injections of thyroid compounds on metabolic rate of dogfish embryos—injection group 1. Solid line, control; dashed line, triiodothyroacetic acid (10 μg.); dotted line, l-thyroxine (10 μg.).

It may be seen in Figures 1 and 2, and Table III, that the oxygen consumption of control embryos varied throughout the periods of respiratory measurement, but not in any particular pattern. The nature of this variation, whether due to maturational or environmental factors, is not clear. However, whatever the basis for the variation in control respiratory metabolism, the changes were generally gradual. In the first experiment (Fig. 2) oxygen consumption increased gradually through most of the three-week period of observation; in the second experiment (Fig. 3) this variation was, in general, less and showed no such constant trend.

Fortunately, the general variation of the controls was paralleled by the hormone-injected embryos, and in addition, a relative difference from the controls was usually maintained, if it occurred at all. Accordingly, when the results are expressed as per cent of the control oxygen consumption some conclusions appear to be offered (Table III, Fig. 4).

The most potent stimulator of oxygen consumption in these tests was triiodothyroacetic acid (Fig. 4A). Despite all the variations to which such experiments seem to be prone, in all four experiments in which Triac was injected in 10-microgram quantities, it clearly induced an increase in oxygen consumption to maxima 17% to 33% above the control. These maxima were achieved 8 to 10 days after beginning the injections and thereafter oxygen consumption progressively decreased. The larger dose of Triac (100 micrograms) was less effective than the smaller one (Table III).

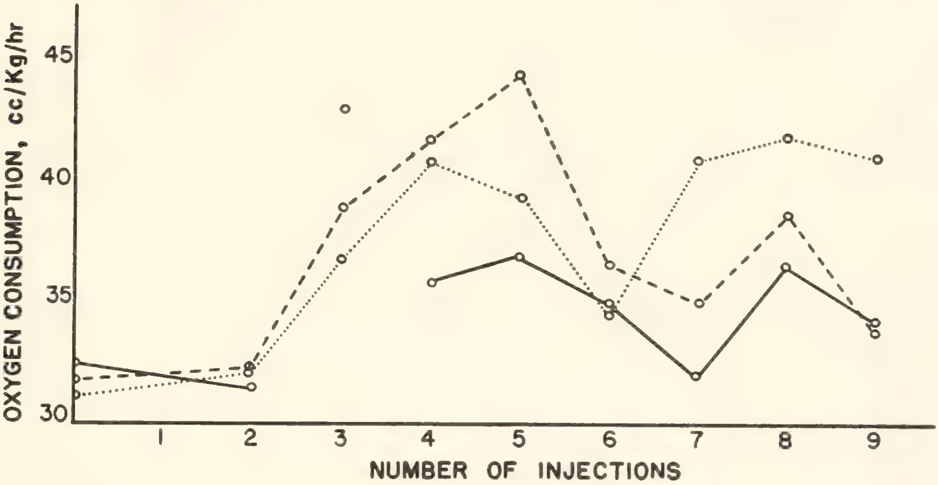

Figure 3. The effects of repeated injections of thyroid compounds on metabolic rate of dogfish embryos—injection group 2. Solid line, control; dashed line, triiodothyroacetic acid (10 μg.); dotted line, l-thyroxine (10 μg.).

Thyroxine, in 10-microgram doses, was not as clearly a stimulator of oxygen consumption in the *Squalus* pups as was Triac. It consistently raised oxygen consumption in two of four experiments (Table III, Fig. 4B) above that of controls, but failed to do so in one, and in another did not produce a significant stimulation until the very end. However, in all thyroxine experiments oxygen consumption was rising at the end of the period of treatment, in comparison with the controls. In one test with 100-microgram quantities of thyroxine a 12% increase above the controls in respiratory metabolism was noted.

Triiodothyronine, in 100-microgram quantities, in all instances stimulated oxygen consumption to levels as high as 18% to 20% above the controls (Table III).

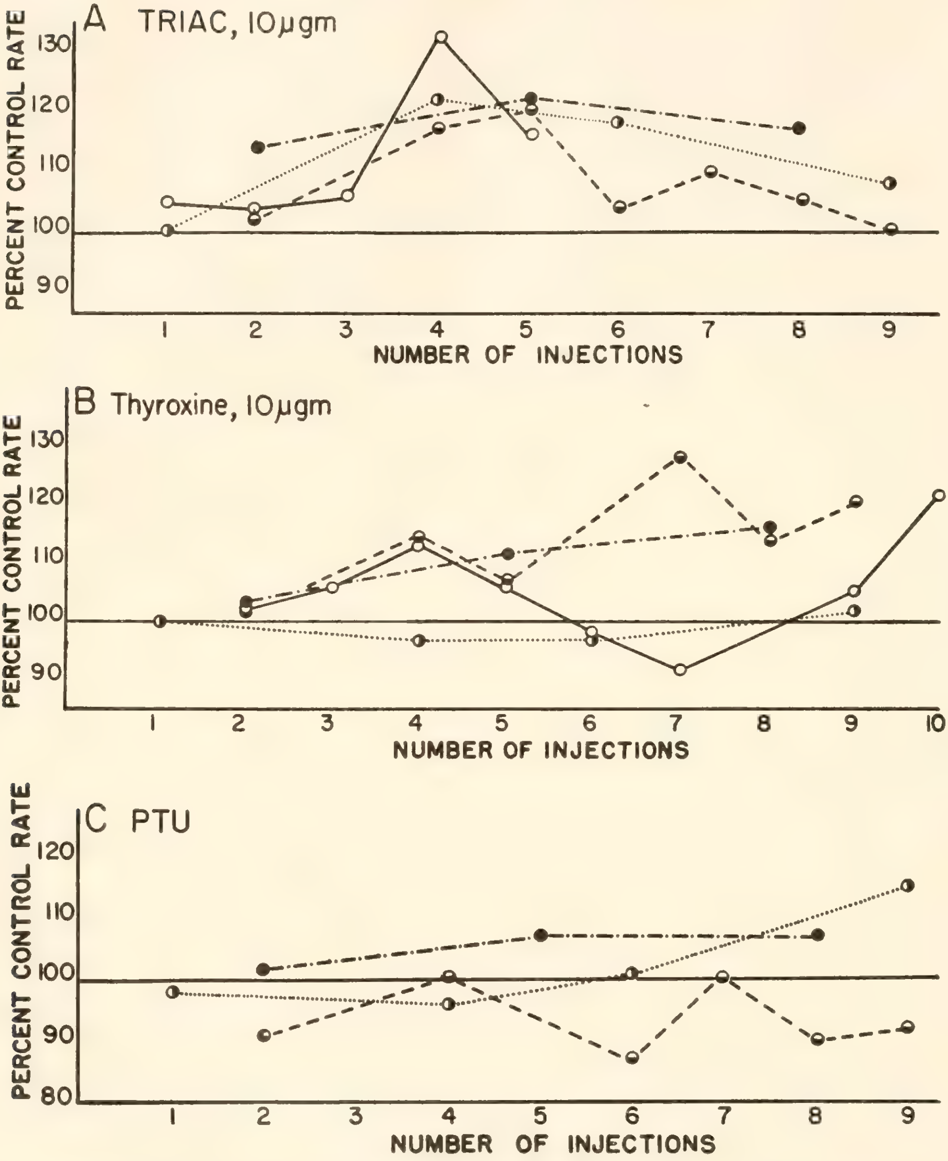

FIGURE 4; A, B, and C. The effects of repeated injections of thyroid compounds on metabolic rate of dogfish embryos. A, triiodothyroacetic acid, 10 μg. each injection; B, l-thyroxine, 10 μg.; C, propylthiouracil, 50 μg. Points represent per cent of the control rate. Open circles, injection group 1; horizontal-barred circles, injection group 2; vertical-barred circles, injection group 3; filled circles, injection group 4.

Propylthiouracil had no particular effect on oxygen consumption relative to the saline-injected controls (Table III, Fig. 4C).

Discussion

Results of this investigation indicate that, under the conditions of these experiments, 10 micrograms of triiodothyroacetic acid (Triac), given on alternate days, have acted as a stimulant of oxygen consumption in the near-term shark embryo (Fig. 4A). The unusual feature of this response is its diminution after 8 to 10 days despite continued injections of the hormone. In laboratory mammals (Foster, Palmer and Leland, 1936) and in clinical use (Means, Lerman and Salter, 1933) the continued administration of thyroxine or crude thyroid preparations is usually accompanied by a sustained increased respiratory rate. However, even in clinical experience it has been reported (Eppinger and Salter, 1935) that an initial rise in metabolism following a week of treatment with thyroid hormone may be followed by a sharp drop, even though treatment is continued. Thus, this pattern of metabolic response is not unprecedented, and may depend on particular physiological factors involved in the response to thyroid hormone. It is of especial interest that Triac has been found to be about 10 to 25 times as active as thyroxine in stimulating amphibian metamorphosis (Pitt-Rivers and Tata, 1959). A ten-fold larger dose of Triac was no more active than the 10-microgram quantity, and appeared, in fact, less active (Table III).

Thyroxine, in either the 10- or 100-microgram dosage, was less clearly a metabolic stimulant. In two of four experiments animals receiving the 10-microgram dose remained consistently higher than controls in oxygen consumption by some 10% to 20%. In the other two experiments this superiority was either lacking or irregularly variable. However, in all four instances oxygen consumption was rising (relative to the saline-injected controls) at the ends of the experiments (Fig. 4B). This was the most variably effective metabolic stimulant and no explanation can be offered for this variability. Larger doses of thyroxine and triiodothyronine (Table III) produce a 12 to 20% increase in oxygen consumption by 10 to 16 days after beginning the injections of hormone.

Propylthiouracil was neither a stimulant nor depressant for oxygen consumption in four different experiments which lasted about 18 days each. Almost all measures of oxygen consumption in dogfish pups treated with this antithyroid drug were within 10% of the control. It has been reported by Zaks and Zamkova (1952) that thiourea consistently reduces the oxygen consumption of young salmon and sturgeons below that of controls. Chavin and Rossmore (1956), working with thiouracil-treated young goldfish, found no effect on oxygen consumption. Interpretation of results of treatment with antithyroid drugs is always complicated by the fact that they are known to be toxic, even in small doses. Since the absence of a metabolic response to propylthiouracil might mean merely that no hormone is yet produced by the thyroid of these embryonic animals, five of them were injected with radioiodide (5 microcuries) and the rest were fixed for histological examination to investigate this possibility. The 24-hour thyroidal radioiodine uptake varied from 0.25% to 2%, a small but significant degree of accumulation. This compares favorably with thyroid uptakes of about 1.5% found by Gorbman and Waterman (unpublished) in pups of the Atlantic spiny dogfish, *Squalus acanthias*. Vivien and Rechenmann (1954) who also treated shark pups (*Scyliorhinus canicula*) with I^{131}, observed by radioautographic techniques that it is deposited in the thyroid, presumably in protein-bound form. The thyroid tissue examined histologically showed a slight increase in average cell height (about 25%) and "vacuolization" of the colloid. This would appear to indicate that the pituitary-thyroid axis of mutual responsiveness is differentiated in these animals, and that it had responded in the PTU-treated animals to a change in thyroid hormone output by TSH secretion. However, despite this apparent decrease in endogenous thyroid hormone production there was no detectable change in oxygen consumption. It is possible that this decrease in endogenous thyroid hormone, if real, was much smaller in size than the 10 micrograms in the injected dose.

In summary, it may be said that triiodothyroacetic acid has been shown in these experiments to be a temporary stimulant of oxygen consumption in near-term embryos of *Squalus suckleyi*. Triiodothyronine proved slightly less active, and the metabolic stimulation by thyroxine was irregular. The thyroid glands of these animals appeared to be functioning at a low rate, and interruption of this function by propylthiouracil had no demonstrable effect on oxygen consumption.

SUMMARY

1. The oxygen consumption rate of "near-term" pups of the dogfish, *Squalus suckleyi*, was determined at regular intervals during the course of repeated injections of physiological saline solution, thyroid hormones, or of anti-thyroid substances. Up to 10 injections were given on alternate days.

2. Of the compounds tested, triiodothyroacetic acid at a dosage level of 10 micrograms per injection was the most consistent in raising the level of oxygen consumption. The effect, however, was transitory with oxygen consumption rising to a maximum (17% to 33%) level above the saline-injected controls after four injections, thereafter declining slowly to control levels.

3. L-thyroxine at a dosage level of 10 micrograms had a variable effect on oxygen consumption. In two of four experiments the oxygen consumption rate rose irregularly, reaching a level about 20% above the controls after 9–10 injections. In the remaining experiments, there was no clear tendency to remain above the controls.

4. Propylthiouracil, after 9 injections, had no consistent effect on metabolic rate in four experiments.

5. The results are discussed with reference to the possible level of thyroid function in these animals.

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PERMEATION AND MEMBRANE TRANSPORT IN PARASITISM: STUDIES ON A TAPEWORM-ELASMOBRANCH SYMBIOSIS[1]

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It was shown by Read, Simmons and Rothman (1960) that the amino acids L-valine and L-leucine enter the tapeworm, *Calliobothrium verticillatum,* by a process showing adsorption kinetics. The data obtained ruled out simple diffusion but did not permit a definite conclusion as to whether the permeation is a process of active transport. L-valine and L-leucine were each shown to competitively inhibit the entry of the other into the worm. Several other amino acids were shown to inhibit L-valine and L-leucine permeation, but it was not established that these inhibitions were competitive. The studies to be reported have shown that L-valine is actively transported, although further investigation has revealed that when *Calliobothrium* is treated as a member of a symbiotic relationship, the operation of the amino acid entry systems is indeed complex. Appreciation is expressed to Dr. G. Wertheim who lent technical assistance during part of this study, and to the gentlemen of the Supply Department of the Marine Biological Laboratory, Woods Hole, Massachausetts, who furnished many living dogfish.

MATERIALS AND METHODS

The methods of collection and handling of *Calliobothrium* from the dogfish, *Mustelus canis,* were similar to those used in a previous study (Read, Simmons and Rothman, 1960). The salt solution used in handling both worms and host tissues had the following composition: NaCl, 250 mM.; KCl, 4.4 mM.; $CaCl_2$, 5.1 mM.; $MgCl_2$, 2.9 mM.; urea 300 mM.; and tris (hydroxymethyl) amino methane-maleate buffer, 10 mM. (pH 7.2). All incubations were carried out in this medium, with appropriate experimental additions, at 10° C. In preparing tissues for experimental incubation, the worms were washed in several changes of the salt solution and incubated for 60 minutes at 10° C. before an experiment. Host tissues were removed in ice-cold salt solution and, before the experiment, were incubated at 10° C. for 30 minutes in a large volume of salt solution containing 10 mM. glucose. Throughout this period, the tissue was vigorously aerated with a sintered bubbler attached to a small air pump. For experimental incubation, the worms or host tissues were lightly blotted on hard filter paper and transferred to the experimental medium. At the end of an experimental incubation, the worms or host tissues were rinsed by dipping twice in large volumes of the salt solution, blotted quickly on hard filter paper, and placed in a measured volume of 70% ethyl alcohol. It was previously shown that, with occasional shaking, free amino acids are extracted from worm tissues in 50 to 70 per cent alcohol in less than 24 hours (Read, Simmons and Rothman, 1960). In most cases extraction was carried out for over 48 hours but in no case for less than 24 hours. Actually, the concentration in the fluid of the tissues probably comes to equilibrium with the alcohol external to the tissues. The quantity of worm tissue with respect to the volume of the extracting fluid was kept sufficiently low so that an error of less than 1% was introduced by the addition of the worm volume to that of the extracting fluid. Aliquots of alcoholic extracts were used for determination of radioactivity or analysis of amino acids.

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Many of the data are expressed in terms of the alcohol-extracted dry weight of tissue; this was determined by heating the extracted tissue for 5 to 6 hours at 100° C. in tared foil pans. Drying for longer periods produced no significant change in dry weight values. Determinations of the alcohol-extracted dry weight/wet weight for 16 worms at the end of a 60-minute incubation in the salt solution at 10° C. gave a value of 0.302 ± 0.016. Nineteen dogfish gut samples, identical with those used in experiments, gave an alcohol-extracted dry weight/wet weight value of 0.135 ± 0.011 after a 30-minute, aerated incubation in salt solution with glucose at 10° C. Plating of extract samples and determinations of radioactivity were carried out as previously described (Read, Simmons and Rothman, 1960).

Two-dimensional chromatography was carried out by a modification of the method of Levy and Chung (1953), as described by Campbell (1960). One-dimensional chromatograms were prepared on Whatman No. 52 paper using sec-butyl alcohol, formic acid, and water (75:15:10) as the solvent system. Amino acids were quantitatively estimated using the methods of Fowden (1951). Radioautographs of chromatograms were prepared by exposing Eastman "no-screen" x-ray film to the chromatograms after removing the solvent. Histidine was determined by the method of Macpherson (1946). Nitrogen was determined by the micro-Kjeldahl method described by Lang (1958). Other details of methods will be described in context.

EXPERIMENTAL

Further characterization of the amino acid entry systems of Calliobothrium

Several amino acids have been shown to inhibit the penetration of L-valine and L-leucine into *Calliobothrium* (Read, Simmons and Rothman, 1960). In the present study a number of experiments were carried out to determine whether these inhibitions are competitive in nature, and whether there is a reciprocal inhibitory effect of L-valine on penetration of certain of the inhibitory amino acids. An analysis of the inhibitory effects of L-serine, L-threonine, and L-alanine on valine entry showed that the inhibitions indeed are competitive in nature (Table I). Conversely, experimental analysis of the effect of L-valine on the

Table I

Effect of L-serine, L-threonine, and L-alanine on the entry of L-valine into Calliobothrium. *S = concentration of L-valine; V = counts per minute per gram of alcohol-extracted dry tissue; N = number of samples*

Amino acid	Inhibitor	N	1/S	V
L-valine-C14	$5 \times 10^{-3} M$ L-serine	4	200	59,575 ± 3673
		4	500	36,200 ± 2876
		4	1000	18,212 ± 1004
		4	2000	11,515 ± 1529
	$2 \times 10^{-3} M$ L-serine	4	200	96,900 ± 4300
		4	500	41,850 ± 1947
		4	1000	24,300 ± 571
		4	2000	16,812 ± 857
	None	4	200	116,600 ± 10,148
		4	500	61,222 ± 5793
		4	1000	46,700 ± 2200
		4	2000	31,775 ± 1520
L-valine-C14	$5 \times 10^{-3} M$ L-threonine	4	200	65,666 ± 4843
		4	500	36,368 ± 6319
		4	1000	18,769 ± 852
		4	2000	10,042 ± 1102
	$2 \times 10^{-3} M$ L-threonine	4	200	73,450 ± 5576
		4	500	45,300 ± 4529
		4	1000	27,937 ± 1971
		4	2000	16,875 ± 1639
	None	4	200	93,725 ± 7925
		4	500	64,550 ± 5794
		4	1000	40,920 ± 3415
		4	2000	28,300 ± 3706
L-valine-C14	$5 \times 10^{-3} M$ L-alanine	4	200	53,825 ± 3594
		4	500	31,070 ± 2400
		4	1000	22,400 ± 842
		4	2000	10,100 ± 597
	None	4	200	91,550 ± 4132
		4	500	75,902 ± 2926
		4	1000	41,850 ± 4903
		4	2000	28,475 ± 2685

penetration of L-serine demonstrated that valine competitively inhibits the entry of this amino acid (Table II).

Previous studies showed that at a concentration ratio of 2:1, glutamic acid did not inhibit the entry of L-valine. Therefore, it was surprising to find that aspartic acid is an effective inhibitor of L-valine entry. With L-valine at a concentration of 2×10^{-3} *M*, aspartic acid or proline at a concentration of 5×10^{-3} *M*, inhibited L-valine entry an average of 33 and 45%, respectively, in five experiments. A shortage of experimental material prevented a determination of whether or not aspartic acid is a competitive inhibitor.

L-lysine-C14 penetrates *Calliobothrium* at a very low rate. A series of experiments were performed to determine whether L-lysine or L-valine affect the entry of the other. L-lysine entry was not significantly affected by L-valine and L-valine entry was not affected by L-lysine (Table II). The apparent stimulation of L-valine entry by L-lysine at a lysine/valine ratio of 2, previously reported (Read, Simmons and Rothman, 1960), is not considered to be significant in view of the results obtained when this broader range of concentration ratios was examined.

When *Calliobothrium* was incubated for two minutes in 5×10^{-3} *M* L-valine-C14 and subsequently incubated for additional periods in salt medium without

Table II

Effects of certain amino acids on entry of L-serine, L-valine, and L-lysine.
Data presented as in Table I

Amino acid	Inhibitor	N	1/S	V
L-serine-C14	$5 \times 10^{-3} M$ L-valine	4	200	45,600 ± 6111
		4	500	29,500 ± 8201
		4	1000	19,100 ± 1124
		4	2000	11,460 ± 823
	None	4	200	57,433 ± 2441
		4	500	42,150 ± 3300
		4	1000	29,100 ± 629
		4	2000	20,200 ± 4540
L-valine-C14	$5 \times 10^{-3} M$ L-lysine	4	200	105,975 ± 13,192
		4	500	73,050 ± 2563
		4	1000	47,300 ± 3379
		4	2000	32,750 ± 2958
	$2 \times 10^{-3} M$ L-lysine	4	200	97,025 ± 12,604
		4	500	72,025 ± 3620
		4	1000	46,250 ± 2418
		4	2000	31,433 ± 2240
	None	4	200	103,775 ± 14,202
		4	500	61,300 ± 963
		4	1000	47,300 ± 2588
		4	2000	34,050 ± 2952
L-lysine-C14	$5 \times 10^{-3} M$ L-valine	4	200	14,970 ± 1207
		4	500	7552 ± 1965
		4	1000	6247 ± 1406
		4	2000	5410 ± 441
	$2 \times 10^{-3} M$ L-valine	4	200	15,115 ± 3924
		4	500	7997 ± 1534
		4	1000	6713 ± 1408
		4	2000	5290 ± 805
	None	4	200	15,358 ± 1665
		4	500	7930 ± 1262
		4	1000	6232 ± 1836
		4	2000	5142 ± 909

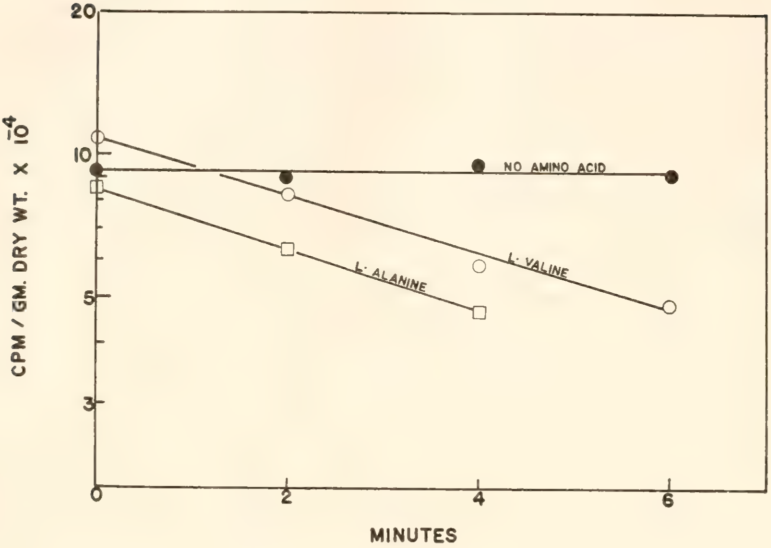

FIGURE 1. Loss of L-valine-C14 from *Calliobothrium* in the presence and absence of unlabeled amino acid in the suspending medium.

amino acid, "leakage" of L-valine from the tissues did not occur. However, when the worm, containing labeled amino acid, was placed in salt medium containing 5×10^{-3} M unlabeled L-valine, a decrease in the C14-labeled valine content of the worm was observed. This was analyzed by varying the time of incubation of labeled worms in medium containing unlabeled amino acid. The loss of labeled amino acid from the tissues followed a decay curve. A similar loss of L-valine-C14 from the worm occurred when the external medium contained unlabeled L-alanine (Fig. 1). In 6 experiments, incubation of worms in 5×10^{-3} M L-valine for 60 minutes had no effect on the entry rate of labeled valine in a subsequent two-minute period.

During the present study attempts were made to determine whether L-valine is concentrated against an electrochemical gradient. Worms were incubated for 40 minutes in L-valine-C14. The medium and the tissues were analyzed for free valine. The concentrations of valine inside and outside the worms are shown in

TABLE III

The accumulation of L-valine by Calliobothrium *against a concentration gradient. Incubation in $9 \times 10^{-3}M$ L-valine for 40 minutes at 10° C.*

Sample	Final concentration in medium µM valine/ml.	Final concentration in worms µM valine/g. water	Worm/Medium
1	6.5	16.9	2.6
2	5.5	23.7	4.3
3	6.3	19.2	3.0
4	7.2	21.3	2.9

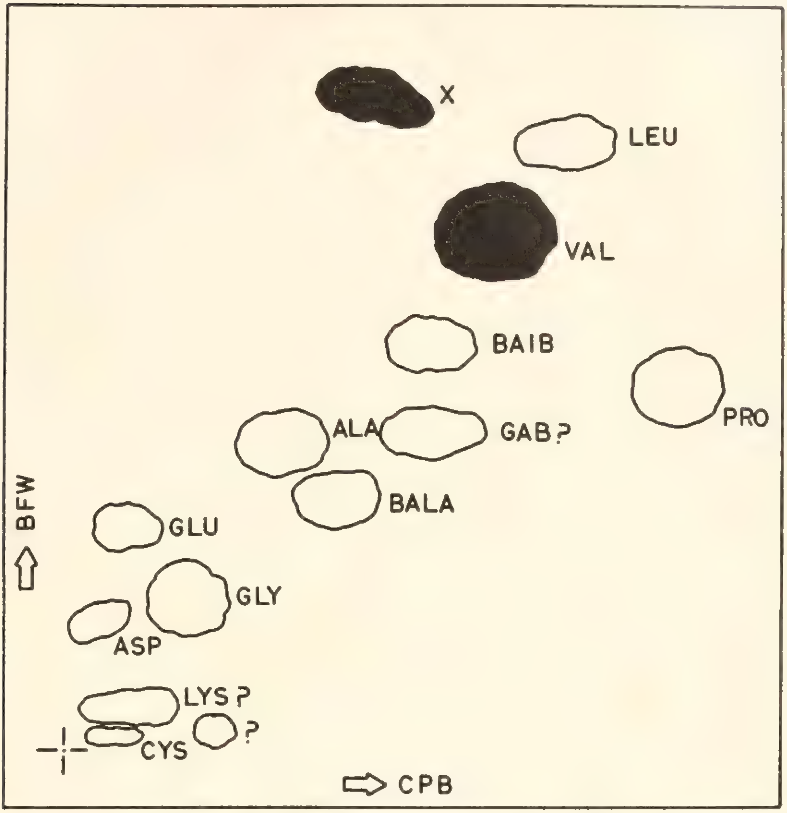

FIGURE 2. Chromatogram of free amino acids extracted from *Calliobothrium* after 40 minutes' incubation in presence of L-valine-C14. Solvent systems were sec-butyl alcohol, formic acid, water (75:15:10) = BFW and meta-cresol, phenol, borate buffer, ph 8.3 (60:30:15) = CPB. Black areas show presence of radioactivity, X being a non-ninhydrin-positive, unidentified metabolite.

Table III. It is apparent that valine is concentrated against a gradient. Radioautographs prepared from the chromatograms of tissue extract from the 40-minute incubations revealed that some L-valine is metabolized in this period. However, L-valine was the only radioactive ninhydrin-positive compound on the chromatograms. A second radioactive spot was present but did not react with ninhydrin. A representative chromatogram is shown in Figure 2. It was not feasible to characterize the unknown metabolite further. On the other hand, radioautographs prepared from 2-dimensional chromatograms of worm extracts from 2-minute incubations in L-valine-C14 revealed a single radioactive spot which proved to be identical with valine by the "fingerprint" method.

The amino acid entry systems of the host gut

Agar *et al.* (1956) differentiated and studied (1) the absorption of amino acids by the rat intestine *in vivo;* (2) the transfer of amino acids from inner to

TABLE IV

Uptake of L-valine-C14 by spiral valve tissue of Mustelus *spiral valve. Incubation in* 5×10^{-3} *M L-valine for 2 minutes at 10° C. Values are counts per minute per gram alcohol-extracted dry weight*

	Spiral No. 2	Spiral No. 6
	80,300	79,300
	85,900	89,600
	93,500	65,100
	74,700	86,500
	79,000	88,900
	87,000	80,600
	88,800	90,000
	82,000	
Mean	83,900	82,857

outer fluids using loops of rat intestine; and (3) the uptake of amino acids by intestinal tissue. In short experiments, the kinetics of the latter two showed rather good agreement, although, as might be expected, a lag was observed in transfer experiments. Since the removal of amino acids from the lumen of the gut by tapeworms and by the host mucosa, rather than transport of amino acids in the

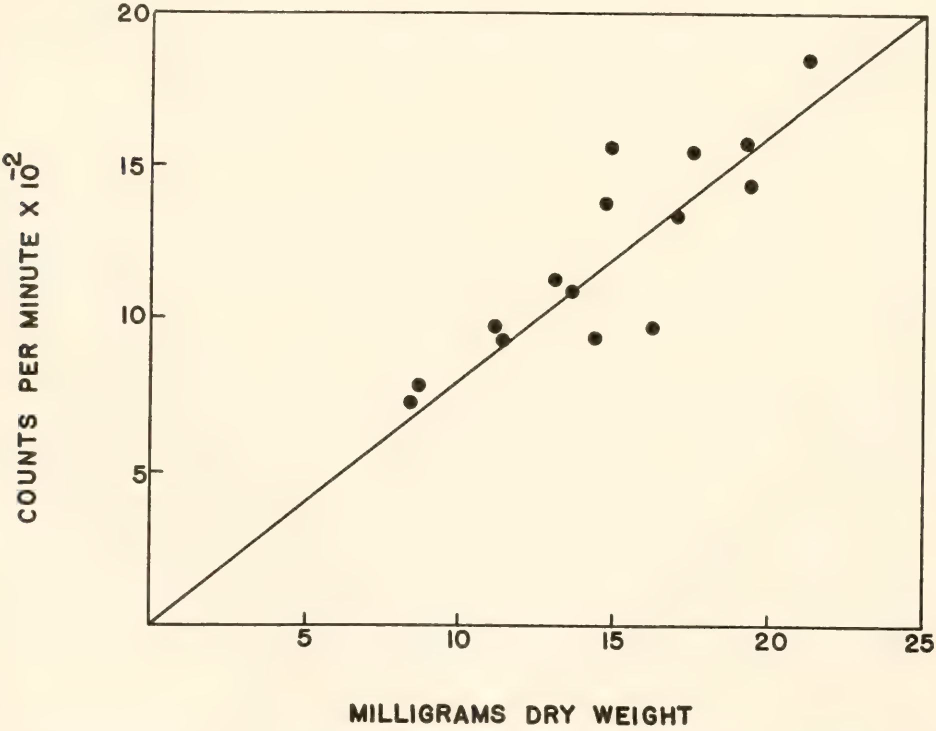

FIGURE 3. The quantity of labeled valine taken up by various amounts of spiral valve tissue of *Mustelus*. Tissue weight is alcohol-extracted dry weight.

extra-intestinal host tissues, is the primary biological aspect to be considered here, experiments to study the uptake of amino acids by dogfish intestinal tissues were carried out. The preparations used were pieces from the lamina of the spiral valve cut approximately 1 cm. square. As many as 30 such pieces can be obtained from the lamina of a single spiral turn in the intestine of a sexually mature dogfish. Most of the experiments to be described were carried out with tissues from the fourth spiral posterior to the pyloric valve, although, as will be shown below, preparations from other spirals would probably yield quite comparable data. *Calliobothrium* is found predominantly in the region of the fourth and fifth spirals. The methods of handling the dogfish tissue were described earlier. It was reasoned that if preparations from different parts of single or separate spiral lamina of the intestine showed consistency in rate of amino acid uptake, with respect to weight of tissue, replicate samples from a single fish could be used in amino acid uptake studies. Initially, therefore, a determination of amino acid uptake was made with tissue samples removed from the second and sixth spirals. Data obtained with tissues from a single fish are presented in Table IV and show remarkably that there is no significant difference in the entry of L-valine into tissues from these two regions of the intestine nor into tissues from different parts of the same lamina. Further, the amount taken up is proportional to the dry weight of tissue used (Fig. 3). These findings showed that multiple sampling for kinetic studies is feasible with these preparations.

TABLE V

Reciprocal inhibition of L-serine and L-valine entry and L-lysine entry into spiral valve tissue of Mustelus canis. *Data presented as in Table I. For experimental details, see text*

Amino acid	Inhibitor	No.	1/S	V
L-serine-C14	None	4	200	42,000 ± 3725
		4	500	28,700 ± 4340
		4	1000	14,900 ± 1859
		4	2000	7695 ± 1236
	5 × 10^{-3}M L-valine	4	500	10,666 ± 1231
		4	1000	6056 ± 586
		4	2000	2966 ± 561
L-valine-C14	None	4	200	68,980 ± 8271
		4	500	35,630 ± 6643
		4	1000	21,996 ± 5036
		4	2000	13,286 ± 1306
	5 × $10^{-3}M$ L-serine	4	200	32,666 ± 2714
		4	1000	15,860 ± 2286
		4	2000	9760 ± 1699
L-lysine-C14	None	4	200	27,293 ± 646
		4	500	17,551 ± 3409
		4	1000	10,800 ± 1184
		4	2000	6575 ± 1197

TABLE VI

Effects of other amino acids on entry of L-valine into spiral valve tissue. V = counts/min./gram alcohol-extracted dry tissue. N = Number of experiments

Inhibitor	N	V
Glycine	4	98,771 ± 6129
L-leucine	4	57,692 ± 3406
L-isoleucine	4	44,326 ± 1267
L-methionine	4	40,253 ± 3294
L-threonine	4	71,655 ± 4029
L-lysine	4	79,363 ± 2326
None	4	91,799 ± 1079

When the concentration of L-valine was varied, it was found that the entry of the amino acid into mucosal tissues follows an adsorption isotherm, and the apparent Michaelis constant for valine entry is about 5×10^{-3}. The addition of unlabeled L-serine at a concentration of 5×10^{-3} produced an inhibition of L-valine entry which was competitive in nature (Table V.) Conversely, L-valine competitively inhibits L-serine entry (Table V) which has an apparent Michaelis constant of about 4.5×10^{-3}.

A number of other amino acids were tested as inhibitors of L-valine entry. Data obtained are summarized in Table VI. It was found that "preloading" the mucosa, by incubating for 40 minutes in non-radioactive L-valine, produced no effect on the subsequent entry rate of L-valine-C14. In balanced salt solution without added non-radioactive amino acid, L-valine does not leak out of the intestinal tissue to a significant extent.

TABLE VII

Relative concentrations of free amino acids in the fluid contents of the spiral valve of Mustelus canis. *All values are related to a valine concentration of 1.00. mg. N = Mg. of alcohol-soluble nitrogen in the sample*

Amino acid	Dogfish No.												$\bar{X} \pm$ S.E.
	1	2	3	4	5	6	7	8	9	10	11	12	
Leucine	1.43	1.46	1.20	1.53	1.43	1.67	1.44	1.15	1.18	1.38	1.61	1.41	1.41 ± 0.047
Phenylalanine	0.77	0.77	0.53	0.71	0.47	0.58	0.62	0.54	0.84	0.57	0.75	0.59	0.64 ± 0.034
Valine	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Tyrosine	0.76	0.91	0.55	0.94	0.43	0.65	0.82	0.63	0.90	0.50	0.75	0.70	0.71 ± 0.049
Alanine	1.35	0.78	0.87	1.94	1.50	1.42	1.65	1.21	1.13	1.25	1.66	1.51	1.36 ± 0.096
Threonine	0.58	0.42	0.35	0.65	0.78	0.49	0.88	0.75	0.83	0.72	1.07	0.84	0.70 ± 0.060
Glutamic	1.98	1.34	1.61	4.24	2.66	3.63	7.65	2.70	2.42	1.99	2.87	2.92	3.00 ± 0.49
Glycine & serine	1.61	1.18	1.72	5.41	2.90	3.12	4.61	2.41	6.26	4.13	3.44	3.14	3.33 ± 0.45
Aspartic	0.95	0.67	1.11	2.47	1.52	1.43	3.06	1.15	2.60	3.50	1.38	3.46	1.94 ± 0.29
Lysine	1.37	1.00	1.70	5.77	7.05	2.27	1.85	1.78	2.04	3.32	6.97	2.44	3.13 ± 0.63
Histidine	0.51	0.48	0.73	—	—	0.77	1.01	0.69	1.09	0.33	1.61	0.51	0.64 ± 0.095
Arginine	0.46	0.57	0.53	—	—	0.39	0.82	0.60	0.86	0.35	0.59	0.33	0.46 ± 0.078
Cysteine	—	—	—	—	0.84	0.70	—	0.52	—	—	—	0.21	0.19 ± 0.090
Beta aminoisobutyric	—	—	—	—	0.18	0.19	—	0.34	—	0.26	—	—	0.081 ± 0.037
Beta alanine	—	—	—	—	—	0.24	1.94	2.21	0.99	0.55	1.44	—	0.62 ± 0.23
mg. N	4.70	1.43	2.27	4.20	7.40	3.35	4.84	3.28	2.60	5.80	3.00	9.40	

Using dogfish gut preparations, attempts were made to show the inhibition of L-histidine uptake by other amino acids in experiments of 30-minute duration. L-histidine was taken up by the tissues and, during the experimental period, molar ratios of histidine in the tissue water/histidine in the external fluid of more than 2 developed. Initial concentration of histidine in external fluid was 1 mM. Addition of L-alanine, L-proline, L-valine, L-serine, or L-aspartic acid at a concentration of 2.5 mM. did not affect L-histidine uptake significantly. Histidine was removed from the medium at the rate of 20 to 40 micromoles per gram dry weight per 30 minutes.

Free amino acids of the intestinal lumen

Samples of the fluid contents of the spiral intestine were collected from a number of dogfish. There was great variation in the nutritional state of these animals. Some were freshly captured and some had been held in captivity for as long as 8 days without food. The samples were taken with a calibrated pipette from the middle portion of the spiral valve of living fish. The measured volume was immediately added to 10 volumes of 70% ethyl alcohol, mixed, and allowed to settle for several days. Free amino acids in the supernatant liquid were quantitatively determined. The analyses are summarized in Table VII. It is evident that there is great variation in the absolute quantity of alcohol-soluble nitrogen and of single amino acid components. However, study of the data shows that, for the most part, the molar ratios of one amino acid to another are strikingly constant. The dicarboxylic acids, aspartic and glutamic acid, show considerable variation, but it may be seen that the ratio aspartic/glutamic is relatively stable. The lysine/valine ratio also showed considerable variation. This may be associated with the relatively low rate at which lysine penetrates the mucosa. The methods used did not allow a clear separation of glycine and serine and these two amino acids were estimated together. However, it was roughly estimated that serine made up about 75 per cent of this value.

Discussion

The demonstration that certain amino acids, which inhibit the entry of L-valine into *Calliobothrium*, do so competitively, as previously shown with L-leucine (Read, Simmons and Rothman, 1960) suggests that this may also be the case with other inhibitory amino acids. Limitations of time and material have not allowed a complete analysis of the inhibitions of valine entry produced by cysteine, methionine, glycine, or proline.

Furthermore, some of the amino acids which do not inhibit the entry of L-valine or L-leucine at a concentration ratio of 2:1 may very well inhibit at higher concentration ratios. D-valine was found to inhibit L-valine entry at a very high D/L ratio but had no significant effect when D-valine/L-valine was 2 (Read, Simmons and Rothman, 1960). Of great interest is the failure of mutual competition between L-lysine and L-valine over a wide range of concentration ratios. Since L-lysine entry appears to follow adsorption kinetics, the lack of interaction with L-valine entry suggests that the two compounds enter at different sites.

The inhibition by other amino acids of L-valine and L-serine entry into the intestinal tissue of the dogfish, shown to be mutually competitive in the case of L-serine and L-valine, is consistent with the observations of others who have reported inhibition of intestinal absorption of single amino acids by other amino acid species in warm-blooded vertebrates (Wiseman, 1955, 1956; Agar *et al.*, 1956).

A difference, however, is observed in the case of L-histidine uptake by dogfish and rat intestinal tissues. Agar *et al.* (1956) found that L-histidine uptake was markedly inhibited by addition of equimolar concentrations of a number of single amino acids. Inhibition was not observed with dogfish tissues when several amino acids were singly added at twice the histidine concentration. Christensen and his co-workers (reviewed by Christensen, 1959) have described reciprocal inhibitions of amino acid uptake with Ehrlich ascites tumor cells and such relationships between amino acids are known to occur with *Neurospora* (Mathieson and Catchside, 1955) and certain bacteria (Cohen and Monod, 1957). It appears to be a very general phenomenon, perhaps as general as the distribution of special membrane mechanisms for amino acid entry. It seems unfortunate that some of the bacterial physiologists have chosen to refer to such entry systems as "permeases," a term implying that there is an entry enzyme. It is to be hoped that a term with such specific connotations is not widely adopted until considerably more understanding of mechanism is attained.

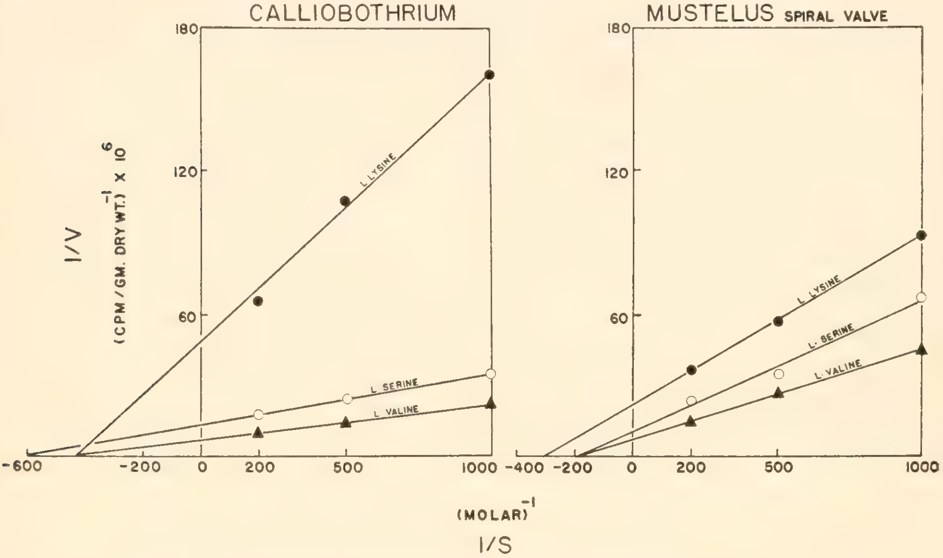

FIGURE 4. A kinetic comparison of the uptake of three amino acids by *Calliobothrium* and *Mustelus* spiral valve tissue.

There are definite differences in the amino acid entry systems of *Calliobothrium* and the dogfish intestinal tissue. In Figure 4 the worm and host tissue are compared with regard to their affinities for three amino acids.

Several years ago the senior author reviewed the literature on the physiology of the small intestine with special reference to its peculiarities as a habitat (Read, 1950). It was concluded that there is a flow of organic compounds, with the notable exception of carbohydrates, from the tissues into the gut lumen and that much of this material is resorbed in areas of the gut distal to the point of secretion. It seemed obvious to infer that many of these materials are available to lumen-dwelling parasites. The present data on the free amino acids in the gut lumen of dogfish in highly variable states of nutrition, and the great stability of molar ratios of these amino acids lend weight to the above concept. As a part of a study of mammalian nutrition, Nasset and his colleagues (1955) demonstrated that molar ratios of free amino acids, one to another, in the small intestine of the dog are astonishingly constant and independent of the composition of protein ingested. The concentration ratios were essentially unchanged in dogs receiving *no* protein by mouth. This has broad implications in considering intestinal parasitism. It reinforces the senior author's argument that in many chemical characteristics, the small intestine represents a relatively stable environment in a particular host. More specifically, it invites inquiry as to what effects constancy of the relative amounts of different amino acids might have on a particular intestinal parasite. It seems plain that if the environment offers a mixture of amino acids or other compounds of nutritional significance, and if these compounds compete with or otherwise affect the entry of one another into the tissues of the parasite, the ratios of amino acids in the mixture will be extremely important in determining whether the nutritional requirements of the parasite can be maintained in a balanced state. The concept emerges that the ratios of nutrients may be *critical* in determining whether or not a given mixture of amino acids will represent a satisfactory food for a parasitic organism such as a tapeworm. Thus, ratios of nutrient concentration may be critical limiting parameters at the *interface* between host and parasite. Further, the ratios of rates of entry of amino acids may be manifestations of an important regulatory system governing the make-up of the amino acid pool in a given worm.

As a homeostatic mechanism of importance in the physiology of the vertebrate, the competitions between amino acids would seem to represent part of a mechanism for regulating the composition of the amino acid mixture entering the portal system and hence the liver. Nasset (1957) has presented evidence that the relative concentrations of amino acids in the small gut are maintained by the secretion of endogenous nitrogenous material which is mixed with the ingesta. It would seem that the regulation of the composition of the amino acid pool for protein synthesis in the vertebrate begins at the mucosa.

While it would appear that the host and the parasite are competing with each other as whole organisms and, from this standpoint, the competition of worm and host should be considered in terms of total absorption by worm and intestine, we may consider highly localized competition in terms of the entry systems for a particular amino acid. If other amino acids affect entry of this amino acid into the mucosa and the worm to differing extents, it is apparent that the concentration ratios of amino acids may undergo alteration in the immediate vicinity of the worm-mucosa system. If the rates involved do not undergo marked change, a new set of concentration ratios should be established. If this is indeed true, it may have wider implications in considering parasitisms in which the most obvious effects on the host are general unthriftiness or ill defined interferences with nutrition. It has not been feasible to study this experimentally in the dogfish-cestode system but it may be practical with other host-parasite combinations more amenable to laboratory control.

It becomes increasingly apparent that the gut should not be considered a space outside the vertebrate body. The rapid changes in the properties of intestinal mucus indicating hydrolysis of components (Hartiala and Grossman, 1952) suggest that secretion, hydrolysis, and resorption must occur constantly. Lumen parasites are in a position to remove from this exocrine-enteric circulation compounds of nutritional value. If the data on rate of entry of individual amino acids into *Calliobothrium* and host intestinal tissue are calculated on the basis of water content, making the assumption that the amino acids are in solution in this water, the tapeworm takes up the amino acids studied at a much higher rate than host intestinal tissue. However, the data on competitions indicate that rates of absorption for single amino acids are not directly applicable to complex mixtures. Study of entry of single components from complex mixtures is obviously required.

SUMMARY

1. The entry of C14-L-valine into the tapeworm, *Calliobothrium verticillatum*, is competitively inhibited by L-serine, L-threonine, and L-alanine. Conversely, L-valine competitively inhibits the entry of C14-L-serine.

2. The entry of C14-L-valine is not significantly affected by L-lysine and, conversely, L-lysine entry is not affected by L-valine.

3. L-valine is concentrated against a gradient by *Calliobothrium* in experiments of 40-minute duration.

4. The entry of C14-L-valine into mucosal tissues of the dogfish host, *Mustelus canis*, is competitively inhibited by L-serine and, conversely, C14-L-serine entry is competitively inhibited by L-valine. L-leucine, L-isoleucine, L-methionine, L-threonine, and L-lysine also inhibit C14-L-valine entry but it has not been shown that inhibition is competitive.

5. In experiments of 30-minute duration, L-histidine uptake by dogfish mucosa was not affected by L-alanine, L-proline, L-valine, L-serine, or L-aspartic acid at the concentrations tested.

6. Quantitative analyses of free amino acids of the dogfish intestinal lumen showed variability in the absolute concentrations but great stability in the relative concentrations.

7. The data are discussed in terms of differences in amino acid entry systems of host and parasite, the significance of stability of amino acid ratios in the nutrition of host and parasite, and the necessity for evaluating host-parasite competitions in terms of entry of single components from complex mixtures.

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PIGMENTED FAT CELLS IN A MUTANT OF DROSOPHILA MELANOGASTER[1]

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The "red cell" mutant of *Drosophila melanogaster* is characterized by the presence of scattered pigmented cells in the thorax and head of the adult fly. This recessive mutant factor, *rc*, is located on the second chromosome, and histological examination of the *rc* phenotype by Jones and Lewis (1957) revealed that the red pigment was localized in granules in some of the pupal fat cells. The expression of the *rc* factor was suppressed in the presence of mutant genes which interrupt the synthesis of the brown pigment of the eye: *vermilion, scarlet,* and *cinnabar*. On the other hand, the mutant gene *brown* which blocks the synthesis of the red component of the normal eye color did not interfere with the expression of the *rc* gene as red cells in the thorax and head of the adult fly. Therefore Jones and Lewis (1957) concluded that the pigment in the red fat cells of *rc* flies is related to the brown pigment component of the eye.

This proposed relationship to insect eye pigments enhances the usefulness of the *rc* mutant as a tool in probing the pigmentation process at a cellular level. The large pupal fat cells offer excellent material for experimental manipulations of individual cells. In addition, the implantation experiments of Beadle (1937a, 1937b) with eye discs and various tissues of *Drosophila* established the fatbody as a source of pigment precursors for the synthesis of brown eye pigment. Preliminary observations of interaction between the *rc* mutant and a mutant strain which develops melanotic masses in the fatbody suggested the present study.

MATERIALS AND METHODS

A stock homozygous for both the *rc* and tu^w factors was made, and all experimental procedures utilized material from this stock. The wild type strain, *Ore-R*, was used to verify the morphological relationships of the various fat masses in the larval stages. The *rc* mutant stock was kindly provided by Dr. E. B. Lewis in 1955, and the *rc* gene has been maintained in our laboratory in combination with the tu^w factor since that time. Melanotic tumorous masses occur in larvae homozygous for the recessive factor, tu^w, located on the second chromosome. Detailed studies of this mutant stock have been reported previously (Wilson *et al.*, 1955; Rizki, 1957).

The $tu^w rc$ stock has been raised on Cream of Wheat medium with Fleischmann's yeast. Timed material for the experiments was collected in the following manner. Adult flies were placed in a half-pint bottle containing a paper teaspoon with Cream of Wheat medium heavily coated with a yeast-honey suspension. A fresh food spoon was placed in the bottle twice daily, and after removal from the bottle the spoons were stored in an incubator at 23°–25° C. Beginning at approximately 20 hours after a spoon had first been placed in the collection bottle, the newly emerged larvae were collected at intervals of one or two hours. These larvae were raised on Cream of Wheat-Fleischmann's yeast medium in crystallizing dishes in the incubator at 23°–25° C. All ages of larvae and pupae are thus counted from the time of eclosion from the egg.

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[1] This investigation was supported by a research grant RG 5285 from the National Institutes of Health, Public Health Service.

In the starvation experiments, larvae were removed from the food dishes at 65 hours of age, rinsed in a saturated solution of NaCl, 2% solution of NaOCl, followed by repeated washing in six changes of distilled water. This procedure removed a considerable proportion of the adhering yeast and food particles. Washed larvae were placed on tissue paper strips (Kleenex brand) moistened with distilled water in petri dishes; paper was also placed under the cover of the dish to prevent larvae from crawling out of the dishes. Care was taken to maintain the paper strip moist without excessive wetting. With each starvation experiment, a group of washed larvae from the same collection period was placed in petri dishes on paper strips to which a thin layer of Cream of Wheat medium and Fleischmann's yeast was added. These larvae served as the control fed material. The larvae removed from food at 65 hours of age generally pupated several hours before those which were left on food. No difference between the percentages of adults emerging in the two groups was noted.

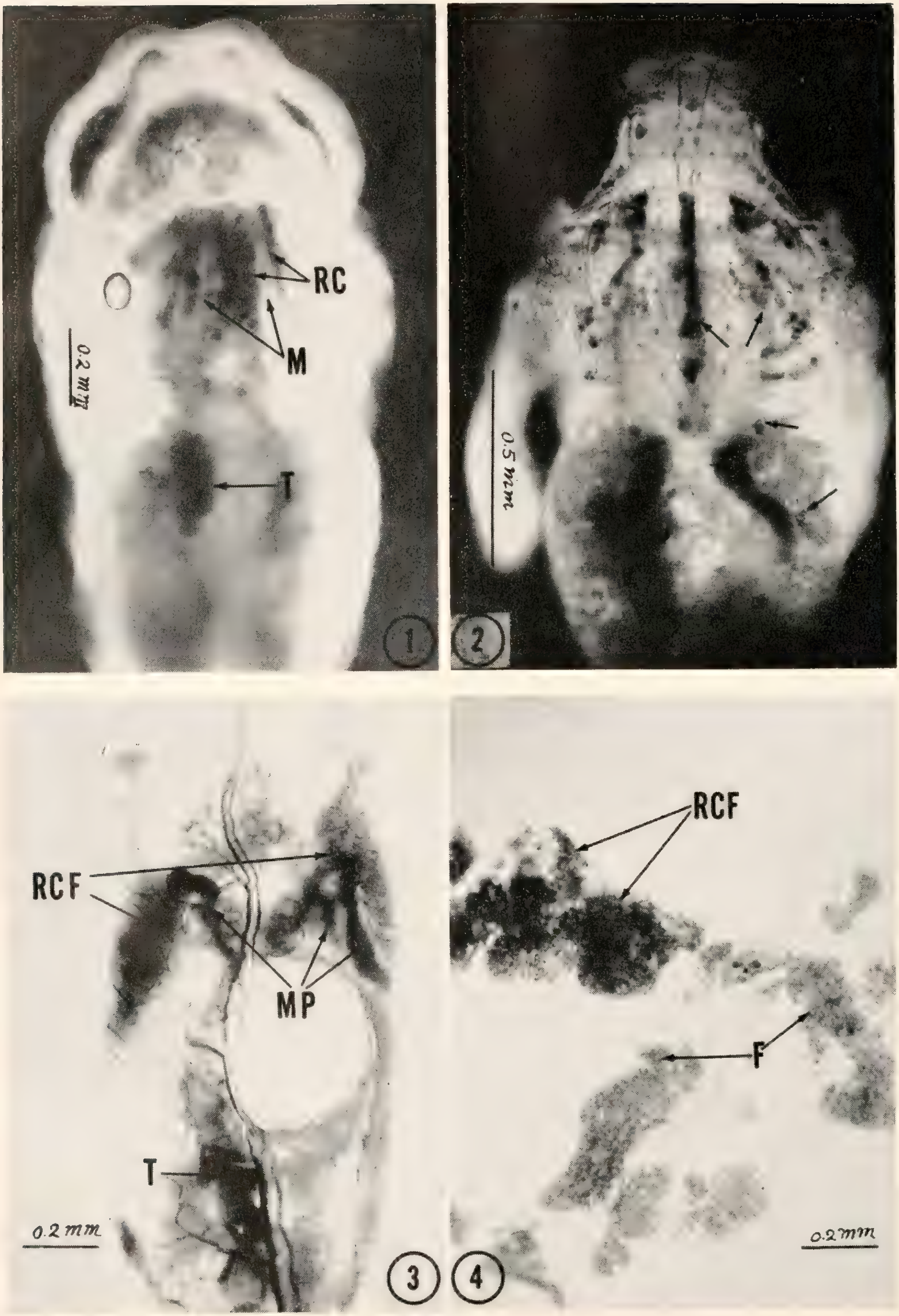
RESULTS

No "red cells" appeared in the $tu^{w}rc$ homozygotes in the stock bottles or in the control fed series. However, the expression of the *rc* factor was 100 per cent if the $tu^{w}rc$ larvae were removed from food at approximately 65 hours of age, that is, during the early third instar. In this case, the mutant pattern appearing in the adult flies was the same as that described by Jones and Lewis (1957) for the *rc* stock. Red-pigmented cells were most abundant in the thorax and head, a few were found in the abdomen, and an occasional pigmented cell was seen in the appendages. In pupae shortly before hatching the red pattern in the thorax was striking. The red-pigmented cells occupied the haemocoel spaces between the flight muscles, and a dorsal striped pattern in the thorax was the result of alternating accumulations of red cells and regions of the muscle insertions (Figs. 1 and 2).

The *rc* factor exhibited no apparent influence on the development of melanotic masses in the posterior fatbody, the phenotypic character of the tu^{w} factor. The penetrance of the tu^{w} factor varied between 90% and 95% during the course of the present investigation; however, the *rc* phenotype was expressed in $tu^{w}rc$ flies which had been starved during development whether melanotic tumors were visible or not.

Control fed larvae and starved larvae were examined at timed intervals after the beginning of the starvation period at age 65 hours. Starved larvae generally pupated two or three hours earlier than larvae which were feeding. No morphological differences were noted between the two groups of larvae until after pupation. During the early period of tanning of the puparium, a slightly yellow tinge was noticeable in the anteriormost fat masses of starved $tu^{w}rc$ pupae. This color

Plate I


RC
M
0.2 mm
T
1
2
0.5 mm
RCF
MP
T
0.2 mm
3
4
RCF
F
0.2 mm

Figures 1–4.

was visible through the puparium which was still very light, but removal of this covering was necessary to reveal the extent of the coloring. Figure 9 is a camera lucida drawing of a starved $tu^{w}rc$ pupa showing the location of the yellow masses underneath the puparium, and a photograph of a specimen removed from the puparium shortly after the pupal molt is labelled Figure 3. Dissection of pupae showed that the yellow color was localized in the most anterior pair of dorsal fat masses just posterior to the cerebral hemisphere, as well as those fat masses which are lateral to this first region and extend ventrally where they join in a commissure. From the ventral side of each of these extensions, a strand of fat cells passes anteriorly where they adhere to the paired salivary glands. The fat masses just described are the only fat cells which developed the yellow pigment. Larvae of the *Ore-R* strain have been dissected to establish morphological correlations of the fat masses in the mutant strain with the fat masses in the normal material. Dissection of the larvae of the wild type strain was considerably easier since manipulation of the fat masses in the starved $tu^{w}rc$ larvae tended to loosen the fat cells which did not seem to be held together so compactly as in the normal strain. Starvation also decreased the size of the fatbody cells as compared to fed material. In order to demonstrate the relationship of the intact fat masses, camera lucida drawings of the *Ore-R* strain are given in Figure 10. The common feature of these anterior fat masses is their proximity to the distal ends of the anterior pair of Malpighian tubules.

As pupation progressed the cells of the fatbody became separated from one another, and were thus involved in the extensive reorganization of the body tissues which takes place during metamorphosis. The pigmented cells of the anterior fatbody were redistributed during this process and were found primarily in the head and thorax of the developing pupa. The color of the fat cells in the intact fatbody of starved $tu^{w}rc$ pupae gradually changed from yellow to yellow-brown, and as the scattering of the separated cells occurred, the color deepened, and finally red pigment was apparent. The yellow color in the fatbody appeared before the imaginal discs had everted, and the red pigment in the fat cells preceded the appearance of the red eye color.

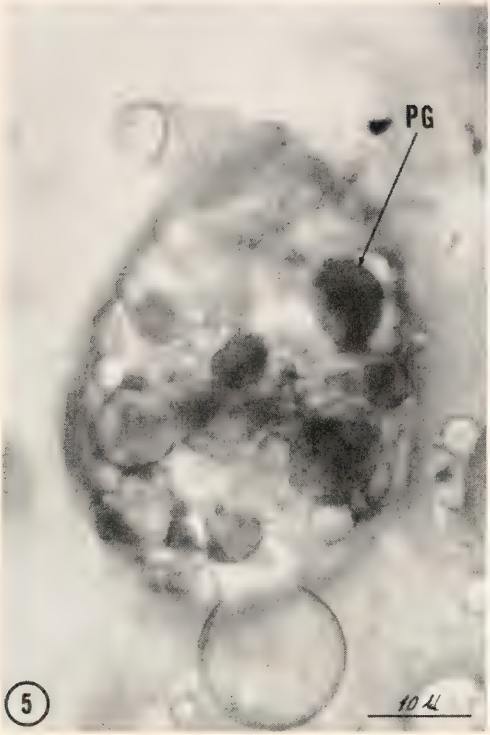
FIGURE 1. Photomicrograph of a $tu^{w}rc$ pupa (starved) removed from the puparium. Red cells, RC, are distributed between the longitudinal muscles, M, of the thorax. A typical melanotic tumor, T, is apparent in the abdomen and two *rc* fat cells are visible on the left margin of the tumor. (Darkfield illumination with a green filter. × 63.)

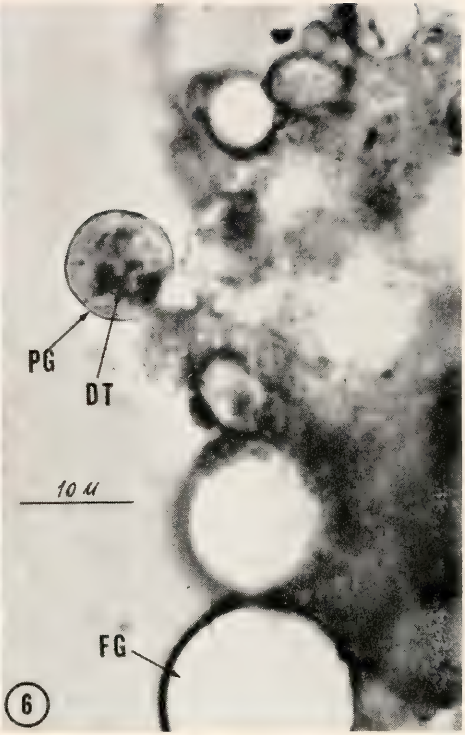
FIGURE 2. A fully formed imago removed from the puparium to show the further dispersion of the red-pigmented fat cells (arrows). Note the retention of *rc* cells in the mid-dorsal region and the further change in the distribution of *rc* cells in the areas not occupied by the insertions of the dorsoventral muscles of flight. Very few *rc* cells are seen among the fat cells of the abdominal region. The eyes are fully pigmented. (Darkfield illumination, green filter, magnification × 60.)

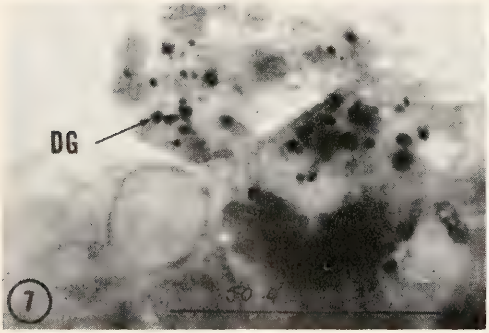
FIGURE 3. A $tu^{w}rc$ (starved) prepupa removed from the pupal case, showing the position of the intact pigmented masses of fat, RCF. Note the intimate association of the anterior pair of Malpighian tubes, MP, with the RCF. The photograph represents the left dorsolateral view of the prepupa so that the left tracheal trunk is apparent and the right tracheal trunk can be visualized as a white band at the lower right margin of the photograph. The dark area under the left tracheal trunk is a melanotic tumor in the caudal fatbody, T. (Transmitted light from a Corning filter #CS 7-59. Magnification × 63.)

FIGURE 4. The intact fat masses removed from a $tu^{w}rc$ prepupa (starved) showing the difference between pigmented fat masses, RCF, and unpigmented fat masses, F. This fat was treated with potassium metabisulfite to intensify the color. (Corning filter #CS 7-59; × 63.)

PLATE II







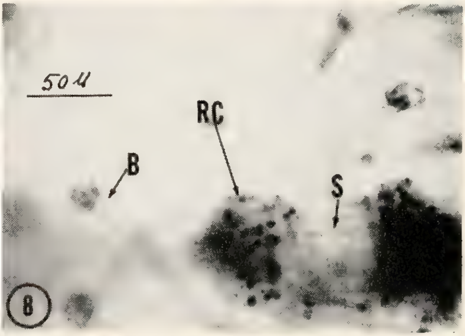


FIGURE 5. A pigmented fat cell isolated from an early prepupa ($tu^{w}rc$ starved) showing the intracellular distribution of pigment globules, PG. (Corning filter #CS 7-59.)

FIGURE 6. A pigment globule removed from the cytoplasm of a red cell from a $tu^{w}rc$ prepupa (starved) showing the threadlike, DT, internal structures characteristic of these globules. A fat droplet is indicated at FG. (Corning filter #CS 7-59.)

FIGURE 7. The *rc* fat cells as seen through the body wall of a $tu^{w}rc$ starved pupa corresponding in age to that given in Figure 1. Note the appearance of pigment in granular form. (Darkfield illumination, green filter.)

FIGURE 8. Granular appearance of pigmented inclusions in a $tu^{w}rc$ starved imago corresponding in age to that in Figure 2. Bristles, B, and setae, S, are visible in this photograph of a red cell as seen through the body wall. (Darkfield illumination, green filter.)

Both the red and brown pigment extracts from the eye of *Drosophila* are altered by oxidation and reduction (Ephrussi, 1942). It seemed desirable to determine whether the yellow pigment in the fat cells would undergo any changes *in vitro*, and a search was undertaken for conditions which might cause such an alteration. Anterior fat masses which had become yellow were dissected from starved pupae in Waddington Ringer-10% glucose. The various reagents to be tested were then added to this medium. The reducing rinse which had been prepared for use in the Feulgen reaction proved most satisfactory. It was then found convenient to add a few crystals of potassium metabisulfite to the drop of glucose-Ringer containing the isolated fat masses while they were under microscopic observation on a white porcelain plate. The color of the fat cells showed a change from yellow to red within a minute. Figure 4 is a photograph of the isolated fat mass in which the *rc* pigment had been intensified in this manner.

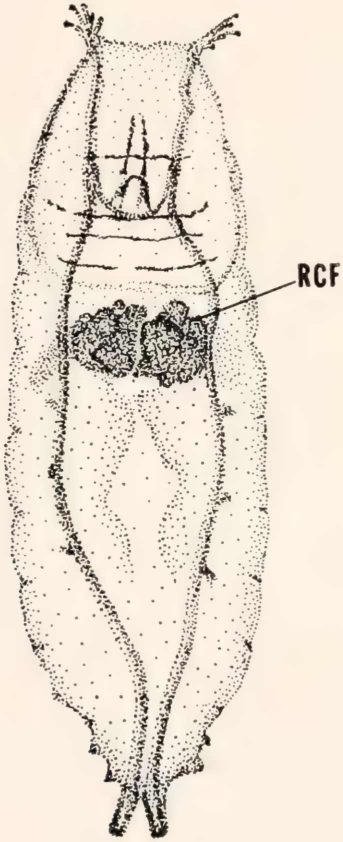


FIGURE 9. Camera lucida drawing of a dorsal view of a $tu^{w}rc$ (starved) prepupa within the puparium illustrating the position of the brownish yellow anterior fat mass, RCF.

The development of pigment in the starved $tu^{w}rc$ flies has been followed at the cellular level. Isolated cells from the anterior fatbodies of young pupae contained numerous yellow cytoplasmic globules. These globules were distinguishable from the fat droplets of the cells which are highly refractile and always spherical in fresh preparations. Isolated cells have been examined with darkfield illumination as well as brightfield illumination, and in addition, the use of a blue Corning Filter No. CS 7-59 proved most satisfactory for studying the cytoplasmic inclusions in the fat cells. With this filter, all yellow objects appeared bright red. In early $tu^w rc$ pupae (after starvation), this included the lightly tanned cuticle, isolated cells from melanotic masses, the granular structures in the Malpighian tubules,

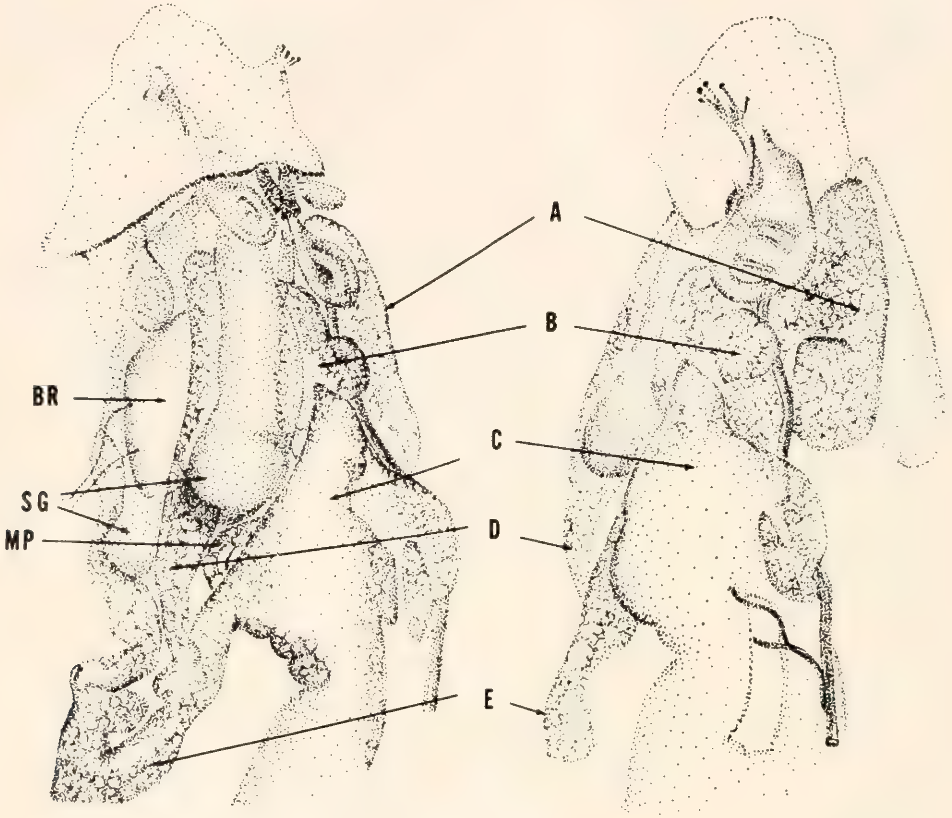


FIGURE 10. Camera lucida drawings of a dissection of a late third instar larva of the *Ore-R* strain showing the relative positions of the fat masses; the salivary glands, SG; brain, BR; Malpighian tubes, MP. This specimen has been stained with Oil Red O. The diagram on the left is the ventrolateral view, and the same dissection has been turned over to show the lateral view of the fat masses in the drawing on the right. A, B, C, D, E are the regions of the fatbody which become pigmented in starved $tu^w rc$ pupae. A, dorsal pair of fat masses; B, C, lateral fat masses; D, fat mass attached to salivary gland; E, fat cells forming the ventral commissure between the right and left masses of fat. Only the anterior region of C becomes pigmented. The club-shaped structures with concentric rings are the imaginal discs and the long tubular structure with branches is the tracheal trunk.

and the yellow globules in the *rc* fat cells. The yellow globules showed a definite threadlike internal structure when examined with this filter, while no structure was discernible in the fat droplets (Figs. 5 and 6). Fat cells from the more posterior regions of the pupa, *i.e.*, cells other than the fatbody cells involved in the expression of the *rc* phenotype, contained similar cytoplasmic inclusions in addition to the fat droplets. Whether these inclusions are structurally and functionally the same as those globules which become pigmented in the *rc* cells remains to be examined. The cells containing the yellow globules were placed on a slide in Waddington-10% glucose solution and several crystals of potassium metabisulfite were added under the coverslip in the vicinity of the cells. The yellow inclusions became red in color under these conditions.

After the pigmented fat cells had become scattered throughout the thorax and head during the developmental processes occurring in pupal life, the color in the fat cells appeared more intense. In the late pupae and young adults, the pigmented structures in these cells were more granular in appearance (Figs. 7 and 8).

DISCUSSION

Two types of pigment are found in the eye of *Drosophila*, one brown and the other red, and many mutants are known which affect the production of these pigment components in the eye. Interference with brown pigment production results in a bright red eye color of the type found in the mutants *vermilion*, *cinnabar*, and *scarlet* while the phenotype of the *brown* eye mutant represents an interruption in the biochemical pathways leading to red pigment. The absence of both pigments occurs in the mutant, *white* eye. The literature on brown eye pigments in insects has been reviewed by Ephrussi (1942) Nolte (1952), and Kikkawa (1953). The synthesis of brown pigment proceeds through a pathway involving tryptophan, formylkynurenine, kynurenine, and hydroxykynurenine, and the known eye color mutants are blocks at successive stages in this synthetic chain. Transplantation of imaginal discs of mutant larvae into hosts of different genotypes has shown that in some cases the eye color is autonomous, whereas some mutant eyes do not themselves produce the prerequisites for brown pigment and are dependent upon other sources in the body for these precursors (Beadle and Ephrussi, 1936; Ephrussi, 1942; Ephrussi and Beadle, 1937). The presence of these pigment precursors has been demonstrated in the fatbody and the Malpighian tubules by transplantation experiments (Beadle, 1937a, 1937b). The time during which each of these tissues produced the pigment precursors was dependent upon the stage of development of the donor. Malpighian tubes showed activity through larval life from the earliest stages tested, appearance of active substances in the fatbody was not detected until after pupation, and in the eyes much later during pupal development (Beadle, 1937b; Clancy, 1940).

The pigment granules in the *rc* cells have been shown to be related to the brown eye pigment of *Drosophila*. Jones and Lewis (1957) found that the mutant factors, *vermilion*, *cinnabar* and *scarlet*, which interfere with brown pigment development in the eye, also prevent the formation of pigment in the *rc* cells when each of these mutant factors is combined with the *rc* gene. The mutant factor, *brown*, which blocks the synthesis of red pigment, does not interfere with the expression of the *rc* gene in the fat cells.

The explanation for the suppression of pigment in the fat cells when the *rc* gene is combined with the tu^w factor may not be so direct. In the starvation experiments the penetrance of the tu^w factor was 90%–95%, and no difference in the expression of *rc* was noted between the pupae with melanotic tumors and those that did not develop black masses. One point of comparison is the fact that both mutants have a common domain of expression, *i.e.*, fat cells: *rc*, the anterior fat mass and tu^w, the posterior region of the fatbody. Tryptophan metabolism is not only related to the development of brown eye pigment and protein metabolism, but it also influences the expressivity and the penetrance of various melanotic tumor genes in *Drosophila*. Addition of tryptophan to the medium increases the frequency of melanotic tumors in strains carrying tumor genes (Hartung and Hartnett, 1951; Plaine and Glass, 1955), and Kanehisa (1956a, 1956b) reported an increase in tumor incidence by combining a tumorous factor with eye color genes. The appearance of pigmented fat cells in $tu^w rc$ pupae after starvation parallels the behavior of the *vermilion* mutants which develop brown eye pigment after the larvae have been starved (Beadle *et al.*, 1938). Starvation of *Drosophila* adults results in a reduction in the size of the fatbody, and the reserves of fat and glycogen are rapidly depleted from the fat cells (Wigglesworth, 1949). In many insects the fatbodies may serve as storage sites for excretory products as well as food reserves. Wigglesworth (1942) has shown that starvation of *Aëdes* larvae causes an increase in uric acid vacuoles in the fat cells and these deposits disappear from the cells after the feeding has resumed. The conditions imposed by starvation in the $tu^w rc$ larvae alter the metabolic pattern of the fat cell such that it differentiates as a pigmented cell. A similar effect, of course, is produced in the *rc* mutant under normal feeding conditions. The presence of the tu^w factor may restore the normal metabolic balance in the fat cell such that their phenotype resembles that of the wild type. The expression of the *rc* phenotype is also dependent upon the action of another recessive gene, *lys*, which causes an accumulation of the amino acid, lysine (Grell, 1958). It is thus obvious that the expression of the red cell phenotype is influenced by the interaction of a number of non-allelic genes. One suggestion may be made which will encompass the various aspects of the problem known at the present time. Any modification, genetic or environmental, which influences the normal pattern of protein synthesis will also alter the metabolic pool of various amino acids. Such changes which affect the availability of tryptophan may be reflected in the phenotypic expression of the *rc* pigment.

The larval fat cells of *Drosophila* form organized tissue masses, whereas soon after pupation the fatbody becomes separated into single cells or small clusters of cells. The cells of the caudal fat masses in tu^w larvae which are involved in the production of melanotic tumors in this strain resemble pupal fat cells in their tendency toward smaller cell aggregations and a loss of adherence to neighboring cells (Rizki, 1957). This precocious change in the structure of the caudal fatbody of tu^w larvae, as well as precocious changes in the blood cells, are processes which lead to tumor formation in the caudal fatbody prior to pupation. Therefore the hypothesis was presented that the melanotic masses in this tumorous strain of *Drosophila* represented an upset in the normal timed pattern of events occurring during metamorphosis. Under conditions favoring expression of the *rc* gene, the

tu^w factor influences an earlier appearance of red pigment in the anterior fat cells. This pigmentation in the *rc* mutant is not apparent until after the fat cells become isolated and scattered during the pupal stage (Jones and Lewis, 1957). However, the combination of *rc* with the tu^w factor has shifted the time of development of the red pigment to a stage preceding this dispersion of the fat cells. Although no obvious explanation for the distribution of the red fat cells among other nonpigmented fat cells existed in the *rc* strain, the morphological relationship of all the red cells in larval development becomes apparent in the tu^w*rc* starved material. A measure of the dispersion of the cells of the anterior fat masses during early pupation is provided in this case by a mutant cytoplasmic marker. The localization of mutant characteristics in the tu^w caudal fat masses and the anterior *rc* fat cells suggests that the cells of various regions of the fatbody may differ in their developmental physiology. It is interesting to note that the cellular ecology of the anterior and the posterior fatbody includes a common feature: the *rc* fat cells are intimately associated with the distal ends of the anterior pair of Malpighian tubules, and the tu^w fat cells encircle the distal ends of the posterior Malpighian tubules.

SUMMARY

1. Cytoplasmic pigment granules are found in some of the fat cells of the recessive mutant, *rc*, of *Drosophila melanogaster*. These scattered red fat cells are located chiefly in the thorax and head, but a few occur in the abdomen and appendages of the adult. Using genetic methods it had been shown previously that these pigment accumulations are related to the synthesis of the brown eye pigment of this insect (Jones and Lewis, 1957).

2. The pigmentation in the *rc* fat cells is suppressed when the *rc* gene is combined with the recessive factor, tu^w. This combination, however, in no way alters the expression of the characteristic pattern of tu^w as revealed by the presence of melanotic tumors in the caudal fat masses of the homozygous tu^w*rc* flies. After a period of larval starvation, the tu^w*rc* flies develop both the red-pigmented fat cells and melanotic tumors. The time of appearance of the *rc* pigment has been shifted under these nutritional and genetic conditions. The cytoplasmic pigment granules appear in the cells originating from the anteriormost section of the larval fatbody which is closely associated with the anterior Malpighian tubules. During the reorganization accompanying metamorphosis from the larval to the adult stage, these cells are redistributed mostly to the thoracic and cephalic regions while a few are found in the abdomen and appendages. An explanation is thus provided for the cytodifferentiation of pigmented and nonpigmented fat cells found side by side in the adult fly.

3. The nature of the pigment granules has been examined in *in vitro* preparations at each of these periods of development, and of particular interest is the internal threadlike structure of these cytoplasmic inclusions during the early stages of pigment formation.

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EXPERIMENTAL STIMULATION OF GAMETOGENESIS IN HYDROIDES DIANTHUS AND PECTEN IRRADIANS DURING THE WINTER[1]

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There is a long list of benthic marine invertebrates found in the Woods Hole, Mass. region that reproduce during the summer months. The majority of the species of organisms suitable for embryological experiments, listed by Costello *et al.* (1957), fall into this category. In addition, the extensive investigations of Redfield and Deevey (1952) have shown that most members of the fouling community attach during the summer months at Woods Hole and in other localities where there is a considerable difference between the summer and winter temperature extremes.

The question arises as to which of the various environmental variables controls reproduction. Hutchins (1947) studied the world-wide distribution of a variety of benthic marine forms and came to the conclusion that the northward extension of the ranges of a number of organisms is regulated by the minimal summer temperature that permits propagation, thus suggesting that elevated temperatures either stimulate gametogenesis or induce spawning.

Experimentally, Townsend (1940) obtained ripe gametes from the sea urchin *Arbacia* by holding specimens in aquaria at a temperature of 18° to 19° C. for one to two months in the late fall and winter. An ample supply of food was provided. Subsequently, Loosanoff and his co-workers have contributed greatly to the field by demonstrating the stimulating effects of elevated temperatures on the development of the gonads of certain commercial mollusks. Loosanoff and Davis (1950) succeeded in bringing the hard clam, *Venus mercenaria*, into reproducing condition during the winter months by gradually raising the temperature over a period of three weeks from that of the natural environment (5.0 to 7.0° C.) to 20° C. They mentioned that the same could be accomplished by placing the clams directly in water at 20° C. but some mortality occurred. Later (1952) these investigators studied the influence of temperature on the maturation of the gonad of the eastern oyster, *Crassostrea virginica*, and demonstrated a logarithmic increase in the rate of ripening of the gametes as temperatures were elevated from 15° C. to 30° C. They also determined that physiologically ripe gametes were formed earlier in the males than in the females under the same conditions. The importance of nutrition on gonad development was indicated by the failure of oysters in "poor" condition, *i.e.*, containing little glycogen, to respond satisfactorily to the thermal stimulus.

Experimental work on the effect of temperature on gonad development of

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other benthic marine invertebrates appears to be lacking. The investigations to be described were undertaken to determine if certain organisms occupying approximately the same geographical range as that of the oyster and the hard clam would respond in a similar fashion. The serpulid polychaete, *Hydroides dianthus* (= *H. hexagonus*) (Verrill), ranges from Cape Cod to Florida (Pratt, 1948) and is found from the low tide mark to depths of several fathoms. It reproduces from the middle of June to the end of October (Grave, 1933) and the ripeness of the gametes may be easily determined by removing the worms from their tubes and observing the products issuing from the nephridiopores (Grave, 1933). The bay scallop, *Pecten irradians* Lamarck, ranges from Cape Cod to Texas (Turner, 1953). It is a hermaphroditic species that spawns from mid-June to mid-August in the Woods Hole region (Belding, 1931). The ripening of the reproductive products may be determined on macroscopic examination by the development of a bright orange color in the ovary at the distal extremity of the visceral mass.

MATERIAL AND METHODS

Specimens of *H. dianthus* were obtained from the supply department of the Marine Biological Laboratory in the middle of December. They had been freshly collected from the adjacent waters where the temperature was approximately 8° C. The specimens were attached to old *Venus* and *Pecten* shells in a community which included *Astrangia*, sulphur sponges and dead barnacle shells. The tubes ranged between 5 and 7 cm. in length, well beyond the size of earliest sexual maturity (Grave, 1933).

Bay scallops were collected from the Eel Pond, Woods Hole, in December and January, in shallow water at low tide. They averaged a little over 3 cm. in the longest dimension and were the young of the year (Belding, 1931). There were no year-and-one-half old specimens in the Eel Pond and those usually found in nearby localities had been almost completely depleted by commercial fishing. Consequently, larger specimens were not available in adequate numbers for experimental work.

At the beginning of the experiments, a number of specimens of *H. dianthus* were removed from their tubes and placed in warm (23° C.) sea water to determine if they would extrude ripe gametes. No gametes were extruded and the worms were preserved in saturated aqueous mercuric chloride with 5% acetic acid for histological study. Similarly a number of scallops were opened and examined for the characteristic orange color of the ripe ovary. None showed this character and a number of appropriate anatomical portions were preserved in the same manner as were the worms.

The worms were subjected to an elevated temperature in aerated still water. Several tube-encrusted shells, containing about two dozen worms, were placed in a non-toxic five-gallon polyethylene container of warmed sea water held at 23° C. by a constant temperature bath. The water was changed every three days and continuously aerated. Suspensions of the planktonic alga, *Phaeodaloctyllum tricornutum* (= *Nitzschia closterium, forma minutissima*), were added daily for food. The amount added was adjusted to the quantity that the worms would just consume in 24 hours. A control was set up and maintained in an identical manner except that the container was placed in a tank supplied by the laboratory sea water system in which the temperature declined slowly from an initial level of 8° C. The illumination of the experimental group and the control group was practically identical. Growth of the tubes was determined in most cases by measuring the new white addition at the mouth beyond the discolored and fouled original tubes. In cases where the tubes were not sufficiently discolored to make new growth clearly distinguishable, a gram of powdered alizarin was added to the sea water and kept in suspension for 24 hours. The worms readily took up the alizarin and combined it with the calcareous material of the new growth, forming a prominent purple ring of alizarin lake. This treatment provided a very satisfactory method of marking the tubes and apparently had no deleterious effect on the worms. Specimens were removed on the third, fifth, seventh, and tenth days, tested for extrusion of gametes and preserved for histological examination. The experiment was repeated four times during December, January and early February.

Preliminary experiments indicated that bay scallops could not be maintained successfully in still water so they were treated in a different manner. In late January a number of specimens were placed in a 3′ × 3′ × 8″ Fiberglas tank supplied with running warmed sea water at a rate of $\frac{1}{2}$ gallon per minute. The apparatus used to warm the water was similar to that described by Loosanoff (1949) but modified to use electric power instead of gas. The temperature was maintained at 23° C. No food was added because the laboratory facilities for rearing phytoplankton were inadequate to supply the vast quantities required in running sea water systems. Consequently the scallops had to subsist on such quantities of food materials as remained in the water after it had passed through the intake line, reserve tank, and lengthy distributing system. A control was set up in a similar manner except that it was supplied with sea water at the ambient temperature which was holding steady at approximately 3° C. The lighting conditions over both groups were identical. Specimens were removed weekly, examined grossly for gonad development and preserved for sectioning.

Results

Hydroides dianthus

None of the worms shed gametes when first obtained, nor did any taken from the control group which was maintained at a temperature approximating that of the natural environment at any subsequent time. In the experimental group which was maintained at 23° C., gametes were first obtained after seven days of warming. Males shed copious quantities of reproductive material consisting largely of spermatocytes and spermatids with a few tailed spermatozoa. By this time, females shed numerous eggs which were very much smaller than normal size and could not be fertilized. On the tenth day all males tested produced normal spermatozoa and the females shed eggs, the majority of which were of normal size. These eggs were successfully fertilized, underwent normal cleavage according to the usual time schedule (Costello *et al.*, 1957), and produced vigorous trochophores. During the ten days of warming all the worms increased the length of their tubes from three to five millimeters, indicating that they were vigorous and healthy. Worms in the control group showed no measurable growth.

The sequence of events during gametogenesis of the tube worm was followed in histological sections. The winter gonad consists of a series of pairs of syncytial masses of germ cells, one pair to each of the abdominal segments. Each segment contains a mass of reproductive material on either side at the ventro-lateral aspect of the wall of the coelom. Figure 1, A is a typical cross-section of a worm pre-

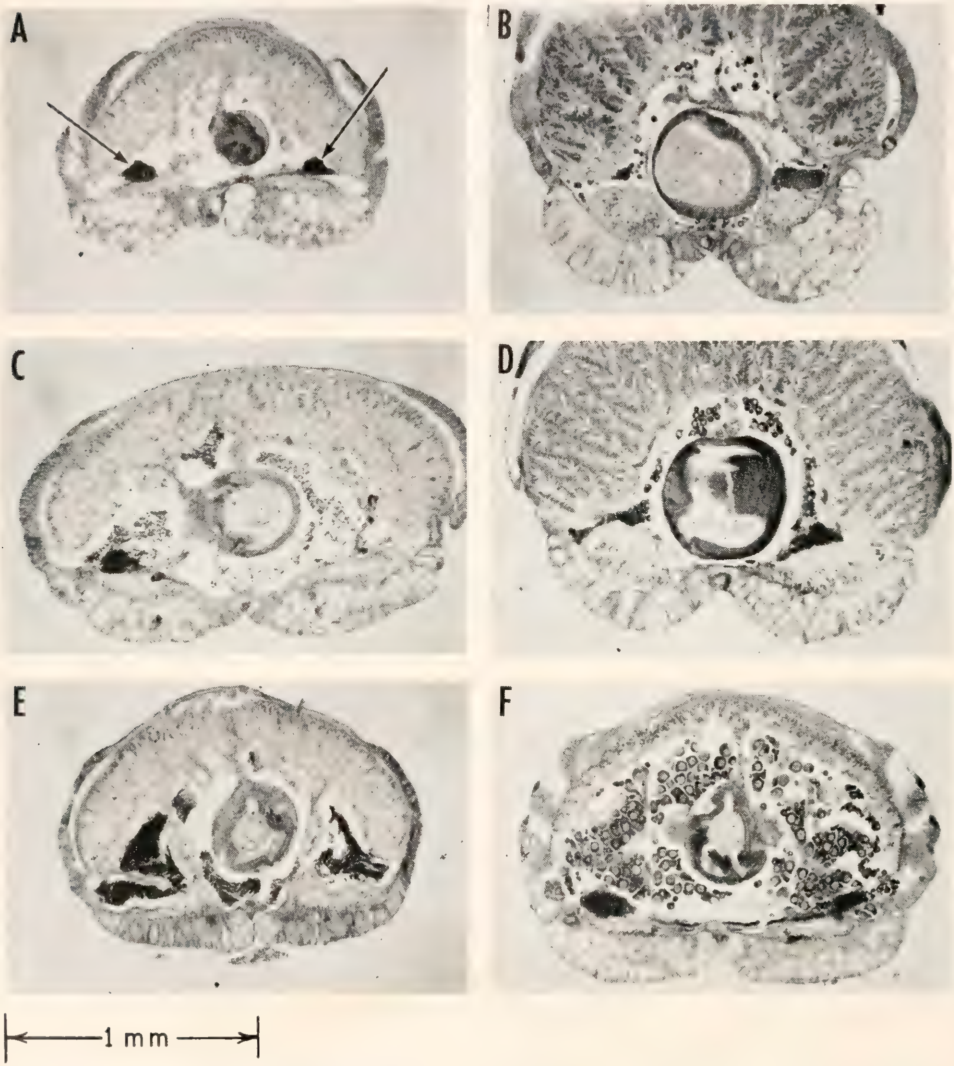

FIGURE 1. Transverse sections of *H. dianthus.* Delafield's haematoxylin and eosin stain. A, Specimen collected in December. Germinal masses indicated by arrows. B, Female held at 23° C. for five days. A few ovocytes free in the coelom. C, Male held at 23° C. for three days. Numerous spermatocytes free in the coelom. D, Female held at 23° C. for seven days. Numerous ovocytes partially grown free in the coelom. E, Male held at 23° C. for ten days. Mature spermatozoa in the coelom. F, Female held at 23° C. for ten days. Mature ova in the coelom.

served in mid-winter. There was little evidence of sexual differentiation at this time although some specimens contained masses with slightly enlarged nuclei, suggestive of primitive ovocytes. All specimens examined contained a few mitotic figures in the germinal masses, indicating that the germinal material was proliferating slowly.

Sexual differentiation became apparent in males after three days of warming. Cells broke away from the germinal masses into the coelom where the maturation divisions took place (Fig. 1, C). This process continued for the next seven days and by the tenth day the coelom was packed with mature spermatozoa, as shown in Figure 1, E.

No significant changes in the female gonads were observed until the fifth day of warming. At this time the nuclei began to enlarge as typical germinal vesicles and a few ovocytes broke free into the coelom (Fig. 1, B). By the seventh day, there were more free ovocytes in the coelom and some had shown appreciable growth (Fig. 1, D). By the tenth day, the coelom was completely filled with ovocytes, the majority of which were of mature size (Fig. 1, F).

There was remarkable uniformity in the rate of development of the gonads in all specimens examined and this was repeated in each of the four experiments run in sequence. At no time did any of the animals taken from the low temperature controls show any evidence of gametogenesis.

Pecten irradians

The reaction of the bay scallops to the elevated temperature was slower and more erratic than that of the tube worm. Approximately 5% died during the course of the experiment. A few specimens failed to show any development of the gametes, and in many, gametogenesis proceeded for a short time and then ceased. The majority of the specimens examined eventually produced tailed spermatozoa and ova which appeared to be structurally mature on histological examination. However, normal spawning did not occur so that physiological maturity could not be determined.

The bay scallop is hermaphroditic. The gonad consists of cylindrical, branching, tubular follicles ramifying through the visceral mass in the blood space surrounding the digestive tract. The follicle wall consists of a single layer of squamous epithelium with germ cells scattered along the inside. Male and female germ cells develop in separate follicles.

Sections of specimens taken from the natural environment in January showed no significant gametogenesis. The follicles of the male gonad as they appeared in sections are shown in Figure 2, A. The follicle walls of the female gonad (Fig. 2, B) appeared to be thicker because of the larger sizes of the ovogonia but there was no evidence of significant development.

Marked changes occurred after the scallops had been subjected to a temperature of 23° C. for one week. Rapid proliferation of the germinal material in the male resulted in a multi-layered lining of the follicles, consisting of spermatogonia and spermatocytes (Fig. 2, C), while the germ cells of the female gonad (Fig. 2, D) enlarged considerably and develop characteristic germinal vesicles with prominent nucleoli (Fig. 2, D.)

At the end of the third week of warming, the ovarian portion of the gonad acquired the orange pigmentation characteristic of the ripe ovary and the testicular portion took on a light, cream color with a plump appearance. Histological sections showed that the male follicles were lined with many layers of spermatozoa with the tails projecting out into the much reduced lumens (Fig. 2, E). The ovarian follicles were much enlarged and were completely filled with ova of mature size

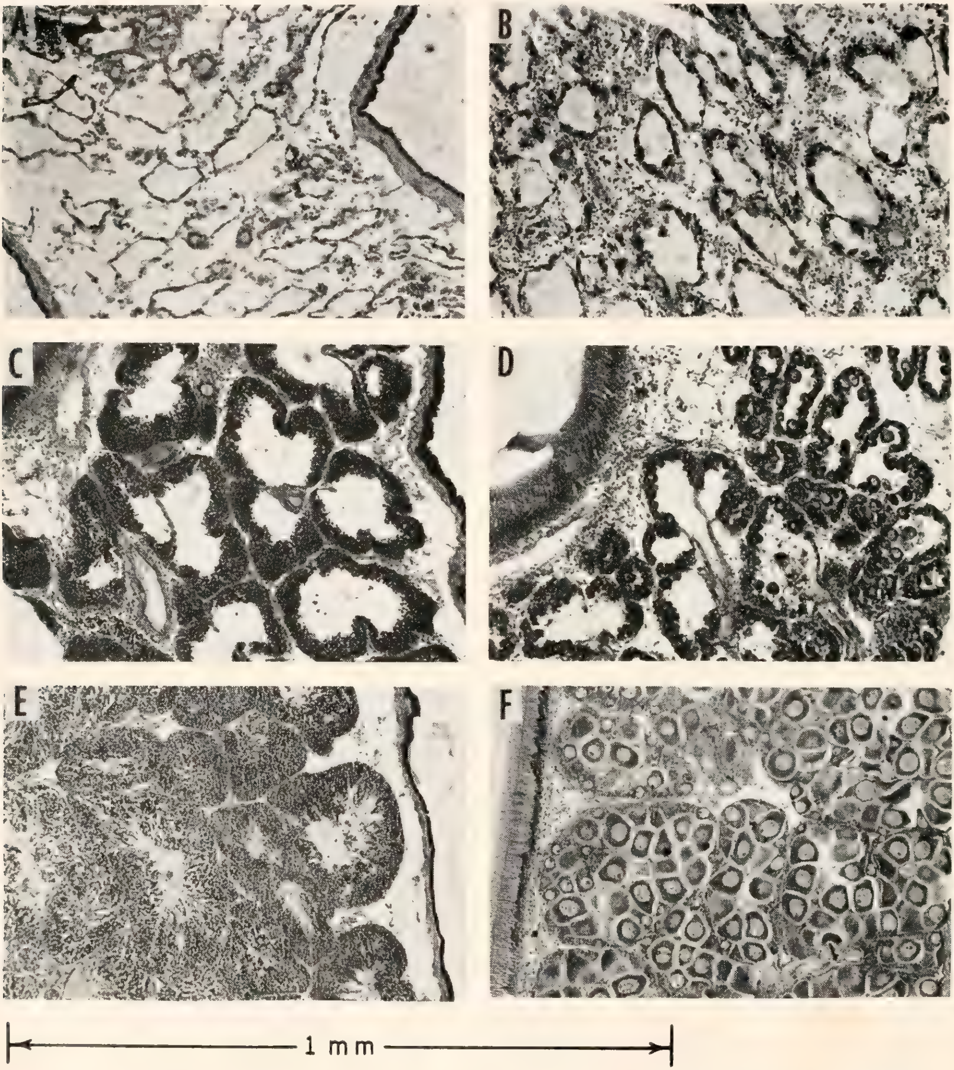

FIGURE 2. Sections of gonads of *P. irradians*. Delafield's haematoxylin and eosin stain. A, Male gonad of specimen collected in January. B, Female gonad of same specimen. C, Male gonad of specimen held at 23° C. for one week. D, Female gonad of the same specimen. E, Male gonad of specimen held at 23° C. for three weeks. F, Female gonad of same specimen.

(Fig. 2, F). None of the specimens taken from the control group showed any evidence of gametogenesis during January and February.

Discussion

It is clear from the foregoing that the tube worm, *Hydroides dianthus*, and the bay scallop, *Pecten irradians*, respond to artificially elevated temperatures during the winter in a manner similar to that shown by many commercial molluscs, as described by Loosanoff, by developing their gametes out of season. All of the tube worms subjected to a temperature approximating that of the natural environment during the normal reproductive period developed ripe gametes within ten days. These underwent normal fertilization, cleavage, and developed into viable larvae.

The response of the bay scallop was somewhat erratic and the development of the gametes was much slower. It is quite probable that the experimental conditions were not entirely satisfactory, particularly in regard to nutrition. Oysters and clams maintained in the laboratory sea water system grow thin and watery after a time, indicating that suspended nutrient material is sparse. Consequently, the scallops held in running sea water at the elevated temperature undoubtedly had to synthesize the reproductive materials from stored substances which may have varied considerably among individuals at the time they were collected. Individuals in which gametogenesis failed to go on to completion were probably those with inadequate supplies of reserve nutrients, similar to the oysters in "poor" condition described by Loosanoff and Davis (1952). In any event it is clear that temperatures approximating those existing during the normal spawning period will stimulate gametogenesis in both *P. irradians* and *H. dianthus* if imposed experimentally during the winter months when the temperature of the natural environment is approaching the seasonal minimum.

Summary

1. Temperatures approximating those existing during the normal summer reproductive period will stimulate gametogenesis in *Hydroides dianthus* and *Pecten irradians* if artificially imposed during the coldest winter months.

2. *H. dianthus* will produce mature gametes in ten days if held in aerated sea water at 23° C. and fed adequate quantities of *Phaeodactyllum tricornutum*.

3. *P. irradians* may produce gametes that appear to be mature on histological examination in three weeks if held in running sea water warmed to 23° C. but in many cases development fails to go to completion.

4. Failure of gametogenesis to reach completion in some specimens of *P. irradians* may be due to inadequate food supply under the conditions of the experiment.

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A NEW SPECIES OF CHIRIDOTEA (CRUSTACEA: ISOPODA) FROM NEW ENGLAND WATERS

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The genus *Chiridotea* is unique to the eastern coastal region of the United States and southeastern Canada. Members of this genus are small (usually < 1 cm.), broad, depressed valviferous forms that inhabit sandy areas and characteristically burrow just beneath the sediment surface. The species described herein constitutes the fourth known species of this genus. Those previously described are: *C. coeca* (Say, 1818); *C. tuftsi* (Stimpson, 1853); and *C. almyra* Bowman, 1955. Included in Bowman's paper is a revision of the generic characteristics of this group. The description of this new form is based on specimens collected on Georges Bank, a relatively shallow portion of the continental shelf east of Massachusetts, U. S. A. These specimens were encountered while processing collections of benthic invertebrates taken by the R/V *Albatross III* and the R/V *Delaware* for the Woods Hole Laboratory, Bureau of Commercial Fisheries, U. S. Fish and Wildlife Service.

Material Examined.—Holotype, adult female with oostegites developed, 7.5 mm. in length, deposited in the U. S. National Museum (Catalogue No. 104282); allotype, adult male 6.0 mm. in length (U.S.N.M. Catalogue No. 104280); paratypes, 1 male 5.0 mm. in length and 1 ovigerous female 6.5 mm. in length (U.S.N.M. Catalogue No. 104281); all type specimens were collected August 6, 1959, by means of a grab-type bottom sampler on Georges Bank at lat. 41° 48′ N., long. 67° 53′ W.; water depth 15 fathoms, sand substrate, bottom water temperature 57.0° F. (R/V *Delaware,* cruise number 59-9, station 21).

One female, 7.5 mm. in length, collected December 7, 1955, at lat. 40° 51′ N., long. 68° 55′ W.; substrate coarse sand, water depth 36 fathoms, bottom water temperature 47.2° F. (R/V *Albatross III,* cruise number 70, collection 3).

One male, 6.5 mm. in length, collected December 14, 1955, at lat. 41° 40′ N., long. 67° 36′ W.; substrate gravelly sand, water depth 28 fathoms, bottom water temperature 45.7° F. (R/V *Albatross III*, cruise number 70, collection 38).

Five females, body lengths 4.0, 5.0, 6.5, 7.5, 7.5 mm., and two males, body lengths 7.0 and 7.0, collected August 24, 1957, at lat. 41° 22′ N., long. 68° 20′ W.; substrate coarse sand, water depth 24 fathoms (R/V *Albatross III*, cruise number 101, station 64).

Three specimens, 4.0, 4.5, 4.5 mm. in length, collected August 24, 1957, at lat. 41° 34′ N., long., 67° 28′ W.; substrate coarse sand, water depth 23 fathoms (R/V *Albatross III,* cruise number 101, station 90).

Diagnosis.—Medium-sized *Chiridotea* with short antennae; flagellum of antenna 2 is much shorter than the peduncle; antenna 1 usually not reaching beyond the peduncle of antenna 2; outer margin of pereion epimeral plates 5–7

FIGURES 1–9. *Chiridotea arenicola* n. sp.

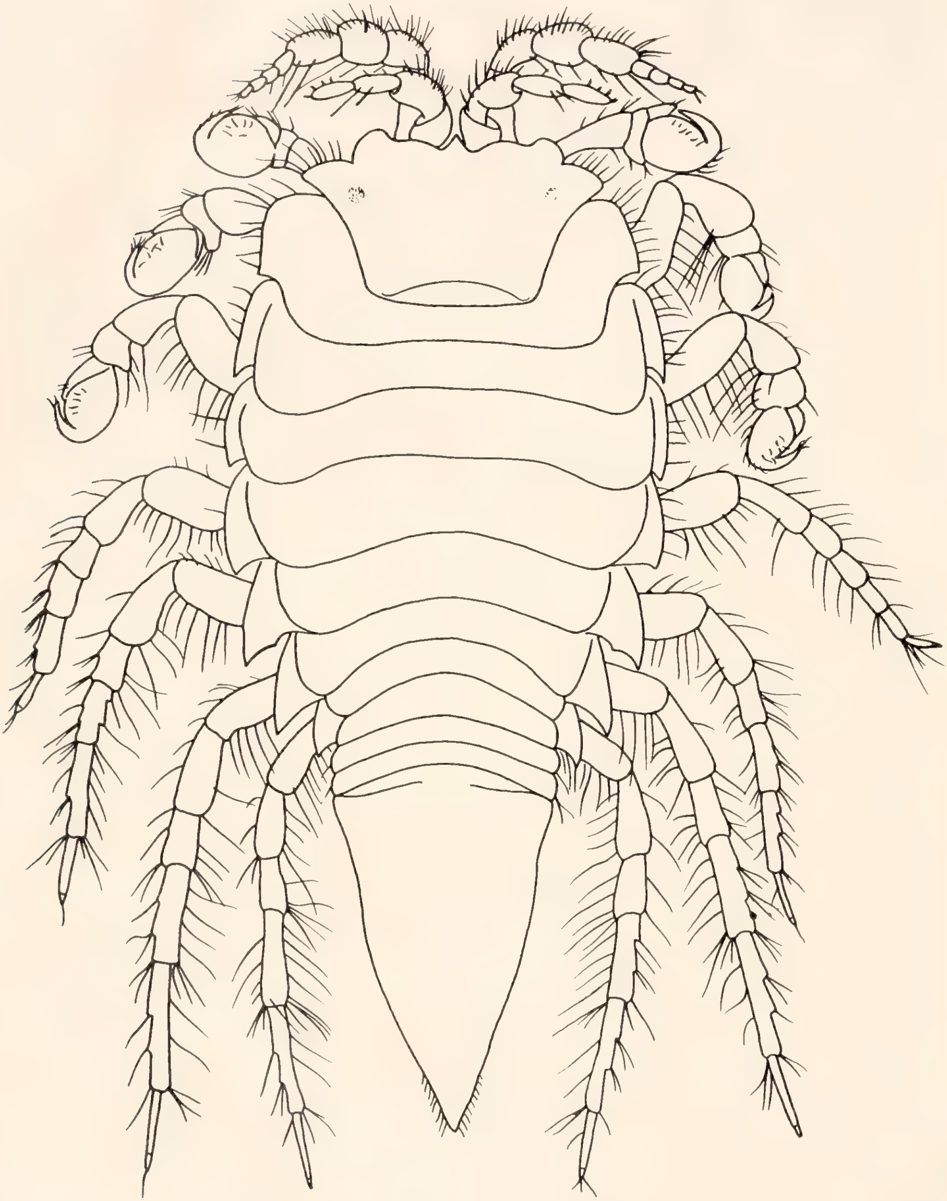

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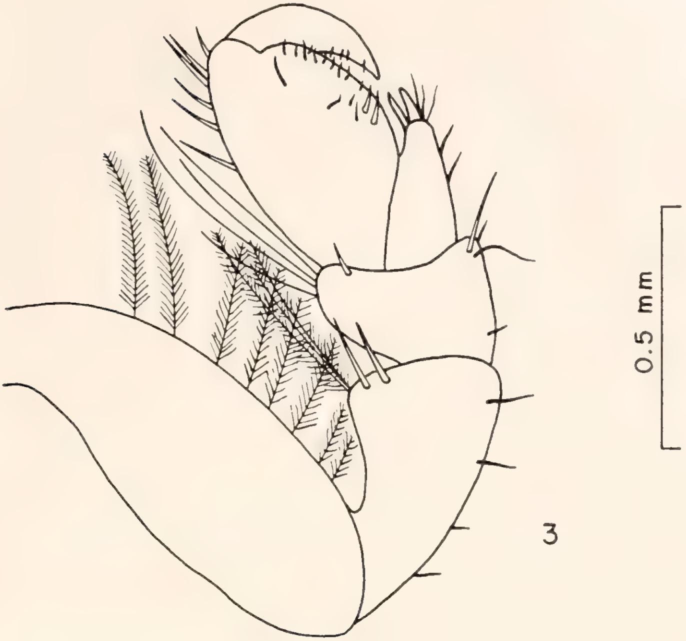
FIGURE 1. Dorsal view of female holotype.



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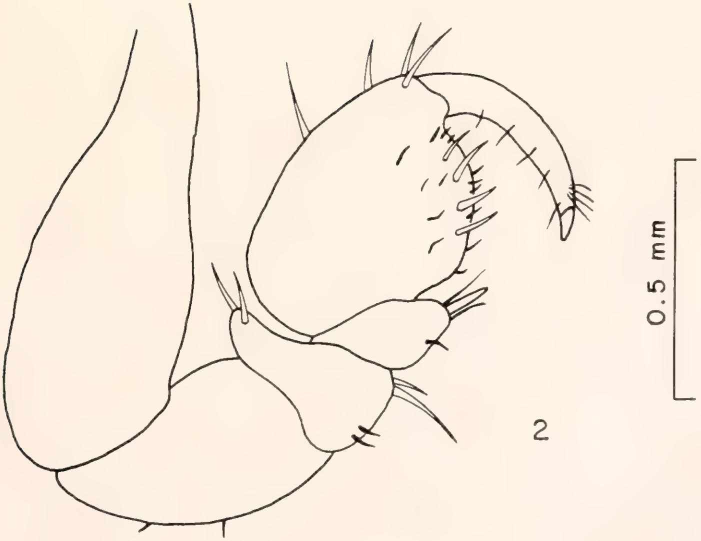

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FIGURE 2. Pereiopod 1, ♂.
FIGURE 3. Pereiopod 2, ♂.

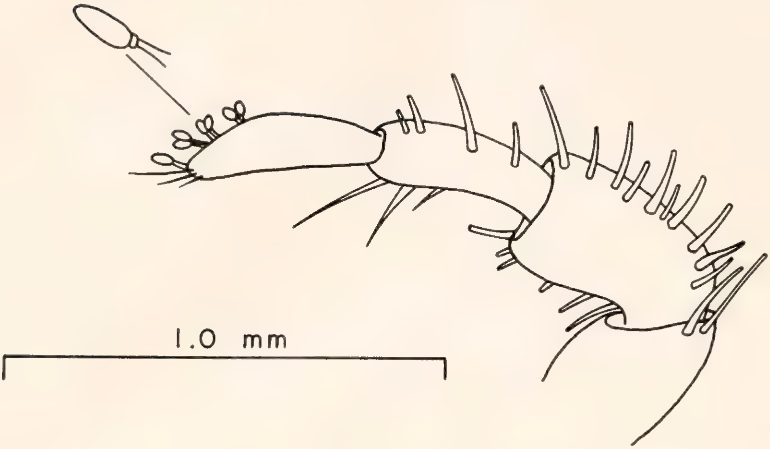

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Figure 4. Antenna 1, ♂.

with relatively few or no setae. This species most closely resembles *C. cocca* (Say) but differs in that it is smaller, the pereiopods are more slender, the body is thinner and less convex dorso-ventrally, the pleotelson is narrower and more evenly tapered, and the anterior lobes of the antero-lateral sections of the head are shorter and have margins devoid of setae. Other differences are mentioned later in this paper under the heading *Discussion*.

Description.—Anterior margin of head broadly and shallowly excavated each side of the rostrum. Lateral anterior projections on the head are rounded or obtusely pointed. Pronounced, but comparatively shallow, V-shaped notch in each antero-lateral margin of the head; margin anterior to the notch is without

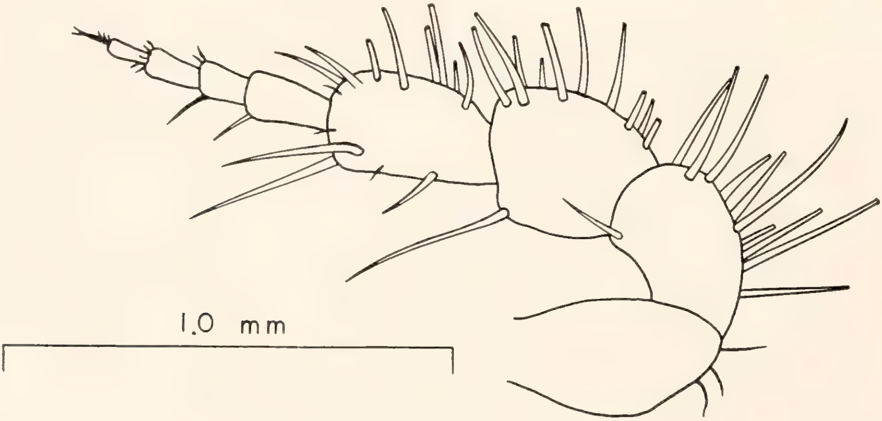

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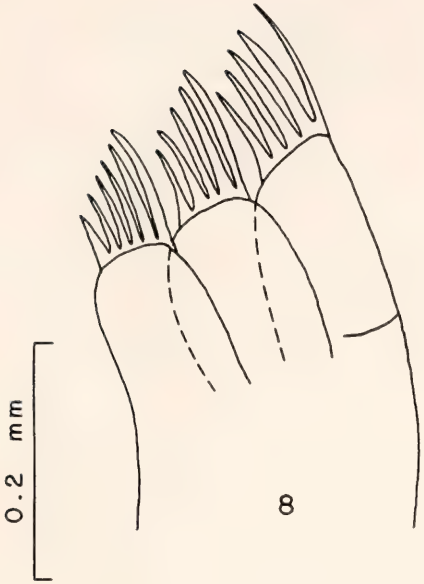
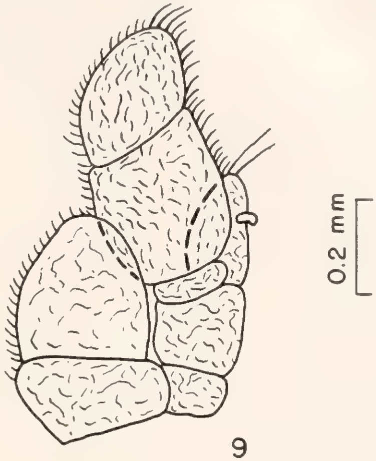
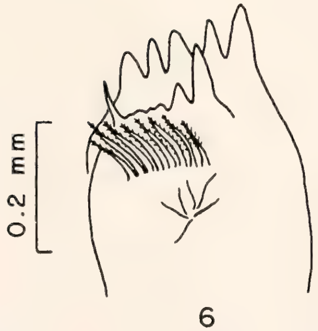
Figure 5. Antenna 2, ♂.







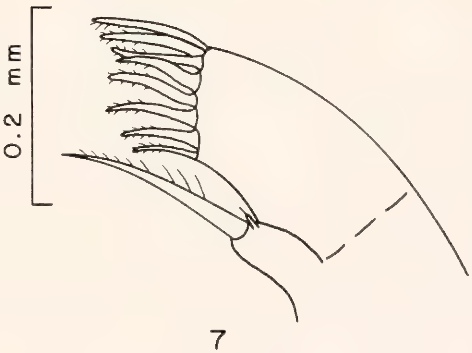


Figure 6. Left mandible, ♂.
Figure 7. Maxilla 1, ♂.
Figure 8. Maxilla 2, ♂.
Figure 9. Maxilliped, ♂.

setae, the margin posterior to the notch has approximately 6 setae. The anterior lobe does not extend outwardly as far as the posterior lobe. Rather small, irregularly rounded compound eyes are located dorsally on the head near the base of the postero-lateral lobe; they are only faintly visible in many of the specimens examined. Epimeral plates distinct on pereion somites *2–7*, their postero-lateral margins acutely produced posteriorly; this projection is more pronounced on plates 5–7 than on plates 2–4. Stout setae fringe the lateral margins of pereion somite 1 and epimeral plates 2–4, and are sparse or absent on plates 5–7. Pleotelson elongate, its length is about 1.6 times its width at the base; sides of the pleotelson taper somewhat irregularly from the base to the apex; lateral margins near the apex are very finely denticulate and beset with setae. General body proportions are: body width 0.43 times body length; abdomen length 0.45 times body length; head length about 0.17 times body length.

Antenna 1 is short, extending only to about the end of the peduncle of antenna 2; the flagellum consists of 1 segment and it usually bears 4 pairs of inflated setae on the anterior margin. Antenna 2 is only slightly longer than antenna 1, its flagellum is made up of 3–5 segments. First segment of the peduncle is expanded; second segment with the distal two-thirds expanded; third segment elongate and slender, approximately equal in length to the flagellum. Maxilliped palp is composed of 3 segments. Maxilla 1 with the inner branch possessing a large, plumose seta and a minute seta. Mandible is without a molar process. Propodus of pereiopod 1 is 1.2 times as long as broad; posterior margin of dactyl 1 bears 4–6 fine setae; a few small setae are present on the external lateral surface of the propodus, near the posterior margin. The posterior (ventral) margin of the carpus of pereiopod 1 is armed with only one stout spine. Pereiopods 1, 2, and 3 are generally similar in conformation and embellishment; likewise, pereiopods 4, 5, 6, and 7 resemble one another.

Color.—Basic color of the body and appendages ranges from light tan or a pinkish hue to nearly white with the integument partially covered with dark chromatophores. The chromatophores are black or deep violet and are distributed somewhat unevenly over the body and the larger exposed appendages. Chromatophores are not evident on the pleopods or the inner mouth parts. In the material studied, much variation was observed in the pigmentation pattern from one specimen to another; however, in all specimens examined the chromatophores are consistently more densely concentrated on the uropods and pleotelson than on other parts of the body. Differences in pigmentation appear to be unrelated to size, sex, or season of capture.

Range.—Georges Bank (east of Massachusetts, U. S. A.) in water depths 15 to 36 fathoms and water temperature 45.7° to 57.0° F.

Discussion.—Shape of the head in *C. arenicola* is quite like that found in *C. almyra*. The posterior portion is comparatively long and somewhat narrowed, and the antero-lateral lobes are rather short. The notch separating the antero-lateral margin into two lobes is shallow, as compared to related species. *C. arenicola* is distinguished from all other species in this genus by the absence of setae on the anterior lobe of the antero-lateral margin of the head.

The propodus of pereiopod 1 in *C. arenicola* is relatively short and wide, similar to that of *C. cocca*, but it lacks the setation on the outer central, lateral surface found in that species. The dactyl of pereiopod 1 has only a few thin setae on the occluded margin; in this feature it resembles *C. almyra* and *C. coeca*.

In *C. arenicola* the size and number of setae on the outer margin of epimeral plates 5–7 varies considerably from one specimen to another; however, these setae are generally shorter and much less numerous in this species than in other members of the genus. A slight sexual dimorphism in this feature is apparent in the few specimens available for study. The setae appear to be longer and more numerous in large males than in adult females and small males.

The shape of the pleotelson of *C. arenicola* is intermediate between that of *C. coeca*, which is broad and tapers irregularly, and that of *C. tuftsi*, which is rather narrow and evenly tapered.

The eye is dark and distinct in some specimens and faint in others. Variation in this characteristic occurs at random among the sexes, specimens of various sizes, and in different collections (some of which have been in preservative for two months and others nearly two years). In the specimens at hand, the eye appears to be proportionately much smaller in *C. arenicola* than that depicted for *C. coeca* by Bousfield (1956).

To my knowledge neither *C. almyra* nor *C. coeca* have been reported from offshore waters, and they have not been observed in our collections of benthic invertebrates from the Georges Bank area. *C. arenicola* and *C. tuftsi* are the only representatives of this genus found in our samples taken during the past few years. Of these two species the latter is far more numerous than *arenicola;* a ratio of approximately 20:1, based on the total number captured. Our records indicate that *C. tuftsi* is rather widely distributed over Georges Bank and nearly always within the 50-fathom isobath. *C. arenicola* seems to be more restricted, having been taken only near the shoals on the north-central part of Georges Bank and on the western end near Great South Channel.

A factor which appears to be important in affecting the distribution of these two species is the particle size composition of the substrate. *C. arenicola* has been taken most frequently from areas where the predominant sediment fraction is a coarse sand (0.5–1.0 mm.). Conversely, *C. tuftsi* is most commonly found where the predominant sediment fraction is a medium sand (0.25–0.5 mm.). Also, there is some evidence that *C. arenicola* either burrows more deeply into the sand or for some other reason is more difficult to evict from the substrate than *C. tuftsi.* This inference is based on two factors: (1) in dredges that normally scrape only the surface of the sea bed, hundreds of *tuftsi* have been caught, compared to only two specimens of *arenicola;* (2) in grab-samplers that usually dig 3 to 6 inches into the sea floor, 10 specimens of *arenicola* have been taken, versus 7 specimens of *tuftsi.*

Some of the more common Crustacea with which *C. arenicola* has been found associated are the amphipods: *Aeginina longicornis* (Kröyer), *Ampelisca spinipes* Boeck, *Amphiporeia virginiana* Shoemaker, *Ericthonius rubricornis* Stimpson, *Haustorius arenarius* (Slabber), *Leptocheirus pinguis* (Stimpson), *Photis dentata* Shoemaker, *Pontogeneia inermis* (Kröyer), *Podoceropsis nitida* (Stimpson), *Sympleustes glaber* (Boeck), and *Tmetonyx nobilis* (Stimpson); the mysid: *Neomysis americana* (Smith); and the decapods: *Cancer borealis*, Stimpson, *Cancer irroratus* Say, *Crangon septemspinosa* Say, *Dichelopandalus leptocerus* (Smith), and *Pagurus acadianus* Benedict. These species are the most abundant crustaceans that were taken in the same bottom-grab samples and dredge hauls with *C. arenicola*. It will be recognized that many of these associates are infauna forms; however, all of the larger species except one are epibenthic. Some species listed above are exceedingly tolerant and are adaptable to diverse environmental features, but others listed here are restricted to very specific types of habitats.

Judging from the information available at this time, it seems likely that *C. arenicola* will be found most closely associated with some of the burrowing amphipods listed above, such as *Amphiporeia, Haustorius,* and *Tmetonyx.*

KEY TO THE SPECIES OF CHIRIDOTEA[1]

1. Flagellum of antenna 2 much shorter than peduncle, segments 5 or less; antenna 1 nearly as long as antenna 2. 2

   Flagellum of antenna 2 longer than peduncle, 8–12 segmented; antenna 1 much shorter than antenna 2. 3

2. Antenna 1 extends beyond peduncle of antenna 2; margin of anterior lobe of the antero-lateral margin of head is setose. .... *C. coeca*

   Antenna 1 does not extend beyond peduncle of antenna 2; margin of anterior lobe of the antero-lateral margin of head is not setose. .... *C. arenicola,* n. sp.

3. Posterior margin of dactyl of pereiopod 1 armed with strong spines; pleotelson narrow, tapering evenly from base to apex. .... *C. tuftsi*

   Posterior margin of dactyl of pereiopod 1 armed with a few fine setae; pleotelson broad, sides nearly parallel at the basal half. .... *C. almyra*

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[1] This key modified after that given by Bowman (1955).

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STUDIES ON THE ISOLATED ISLET TISSUE OF FISH.

III. THE EFFECT OF SUBSTRATES AND INHIBITORS ON THE OXYGEN UPTAKE OF PANCREATIC ISLET SLICES OF TOADFISH[1]

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Little is known about the metabolic activity of islet tissue because in mammalian species this tissue is dispersed into a million or more individual islets of Langerhans, whose total mass approximates only 1% of the pancreatic mass. This makes it very difficult to separate the islet from the acinar tissue. In teleost fish, however, the islet tissue is concentrated into one or more discrete bodies called the principal islets (Diamare, 1899); the acinar tissue, in contrast to that of mammalian species, is dispersed throughout the mesentery and located along the bile duct and within the liver. The toadfish (*Opsanus tau*) was used for this metabolic study of islet tissue because of the accessibility of the islets and the general availability and hardiness of this species.

In a previous study (Lazarow, Cooperstein, Bloomfield and Friz, 1957), the oxygen uptake of islet tissue was measured under varying conditions of pH, tonicity, and electrolyte composition, and the baseline conditions under which maximal respiration of islet tissue occurs were defined. The present paper reports further characterization of the over-all metabolic pathways of islet tissue achieved by studying the effects of specific exogenous substrates and inhibitors on the respiration of islet slices.

MATERIAL AND METHODS

After the toadfish were killed by a blow on the head, the principal islets were dissected from the mesentery and the capsules of the islets were removed. The latter is an important step in the procedure, since any acinar tissue that may be present underneath the capsule, as well as any acinar tissue that may be present within the islet but attached to the capsule by trabeculae, is removed when the capsule is stripped.

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In each experiment a single islet was cut into eight slices and these slices were distributed between the experimental and control groups. The Cartesian diver microrespirometer was used to measure the oxygen uptake of each toadfish slice, in a manner previously described (Lazarow *et al.*, 1957). A modification (Lazarow *et al.*, 1957) of the method of Lowry, Rosebrough, Farr and Randall (1951) was used to measure the protein content of each slice. The metabolic activity was expressed as millimicroliters (mμl.) of oxygen per microgram (μg.) of protein per hour.

The metabolic activity of toadfish liver tissue was also studied in order to compare it with that of toadfish islet tissue. The Warburg manometer was used to make these measurements, and the oxygen uptakes were also expressed in millimicroliters per microgram of protein per hour.

A constant amount of phosphate buffer at pH 7.4 (0.054 M) was present in all of the experiments in this study. The respiration of islet tissue was studied in a medium containing phosphate plus substrate or inhibitor, and the oxygen uptakes thus obtained were compared to those observed in either a medium containing phosphate plus saline or a medium containing phosphate alone.[3]

The various solutions of substrates and inhibitors were prepared at twice the desired final concentration and mixed with an equal volume of 0.108 M phosphate buffer so that the final phosphate concentration was 0.054 M.

RESULTS

Effect of substrates

The substrates studied included glucose, pyruvate (lithium salt), α-ketoglutarate, glutamate, succinate, and isocitrate.

When glucose was added to the medium *in vitro* (Table I) neither islet ($p = 0.21$) nor liver ($p = 0.9$) respiration was altered. It should be noted that the endogenous metabolic activity of liver tissue slices was considerably higher than that of islet tissue.

In a similar series of experiments using pyruvate as the substrate, respiration of islet slices was compared in the following media: (a) a hypotonic medium containing phosphate; (b) an isotonic medium containing phosphate plus NaCl; and (c) a medium containing phosphate plus pyruvate (Table II). If one assumes that the added pyruvate readily enters the islet cells, then the respiration of islet slices in the pyruvate-containing medium should be compared with that in the hypotonic medium because (a) and (c) would be of equal tonicity. When these media are in fact compared, it is noted that pyruvate has no effect on the respiration of islet tissue. If, on the other hand, one assumes that the added pyruvate does *not* enter the islet cells, then media (b) and (c) should be compared since

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[3] Whereas Lazarow *et al.* (1957) reported that optimal respiration of islet slices was observed in a hypotonic medium (0.054 M phosphate), in the present study optimal respiration of islet slices was sometimes observed in an isotonic medium whereas at other times no difference was noted between respiration in hypotonic and isotonic media. Evidence indicates that part of this discrepancy may be due to the varying temperature of the water in which the fish were kept. Further work is now in progress in order to clarify these differences.

TABLE I

Effect of glucose on the respiration of toadfish islet and liver slices

Medium	Tonicity of medium (equiv. NaCl conc.* M/liter)	Islet			Liver		
		No. of deter.	Ave. mμl. O_2/μg. prot./hr.	σ†	No. of deter.	Ave. mμl. O_2/μg. prot./hr.	σ†
0.054 M PO_4	0.0755	17	1.5	0.34	8	2.8	0.91
0.054 M PO_4 + 0.0645 M NaCl	0.140	12	1.9	0.11	8	2.8	0.71
0.054 M PO_4 + 0.129 M glucose	0.140	11	1.9	0.98	9	2.7	0.97

* The concentration of a sodium chloride solution whose tonicity would equal that of the medium used.

† σ = Standard Deviation. The statistical significance of the results (p) was calculated using the following equation:

$$p = \frac{\text{difference}}{\sqrt{\frac{(\sigma_1)^2}{N_1 - 1} + \frac{(\sigma_2)^2}{N_2 - 1}}}$$

where σ_1 and σ_2 are the standard deviations of the control (without substrate) and experimental (with substrate) groups, respectively; N_1 and N_2 are the corresponding number of determinations in each group.

they would be of approximately equal tonicity. Comparison of the respiration in *these* media indicates that pyruvate addition inhibits by 23% ($p = 0.019$).

The addition of α-ketoglutarate (Table III) increased the oxygen uptake of islet slices by approximately 33% when compared with either a hypotonic ($p = 0.07$) or isotonic ($p = .008$) medium.

Table IV shows that a 44% increase in oxygen uptake was observed when islet slices were placed in a phosphate-glutamate medium ($p = 0.016$). This is to be compared to a 30–36% increase observed when toadfish liver slices were placed in the same medium ($p = <0.001$).

Oxygen uptake was also increased when succinate (Table V) was added either to toadfish islet or to toadfish liver tissue. The increase in islet tissue respiration in an isotonic phosphate-succinate medium was 47% ($p = 0.01$) when compared to respiration in an isotonic phosphate-saline medium and 100% ($p = 0.001$) when

TABLE II

Effect of pyruvate on the respiration of toadfish islet slices

Series	Medium	Tonicity of medium (equiv. NaCl conc. M/liter)	No. of deter.	Ave. mμl. O_2/μg. prot./hr.	σ
a	0.054 M PO_4	0.0755	43	1.7	0.70
b	0.054 M PO_4 + 0.0645 M NaCl	0.140	14	2.2	0.69
c	0.054 M PO_4 + 0.040–0.0645 M pyruvate	0.1155–0.140	28	1.7	0.70

TABLE III

Effect of α-ketoglutarate on the respiration of toadfish islet slices

Medium	Tonicity of medium (equiv. NaCl conc. M/liter)	No. of deter.	Ave. mμl. O_2/μg. prot./hr.	σ
0.054 M PO_4	0.0755	13	2.1	0.83
0.054 M PO_4 + 0.0645 M NaCl	0.140	13	2.0	0.60
0.054 M PO_4 + 0.0645 M α-ketoglutarate	0.140	14	2.7	0.84

compared to respiration in the hypotonic medium. The addition of succinate increased the respiration of toadfish liver slices by about 75% ($p = <0.001$).

In contrast to α-ketoglutarate, glutamate, and succinate, another Krebs cycle intermediate, isocitrate, did not stimulate islet respiration (Table VI).

Effect of inhibitors

The use of inhibitors on whole cells, extracts, homogenates, and tissue slices has provided much detailed information concerning the occurrence and components of complex enzyme systems, such as the glycolytic and tricarboxylic acid systems. Various inhibitors were used in this study in order to assess the importance of these enzyme systems for the metabolism of islet tissue.

TABLE IV

Effect of glutamate on the respiration of toadfish islet and liver slices

Medium	Tonicity of medium (equiv. NaCl conc. M/liter)	Islet			Liver		
		No. of deter.	Ave. mμl. O_2/μg. prot./hr.	σ	No. of deter.	Ave. mμl. O_2/μg. prot./hr.	σ
0.054 M PO_4	0.0755	14	1.6	0.66	9	2.2	0.40
0.054 M PO_4 + 0.0645 M NaCl	0.140	11	1.6	0.64	9	2.3	0.23
0.054 M PO_4 + 0.043 M glutamate	0.140	13	2.3	0.78	9	3.0	0.30

Table VII shows the per cent inhibition produced by the various inhibitors. Fluoride added to a phosphate medium was found to inhibit respiration of islet slices by 35% ($p = 0.0063$), iodoacetate inhibited by 53% ($p = <0.001$), malonate by 43% ($p = <0.001$), and azide by 50% ($p = 0.001$).

DISCUSSION

Although the addition of glucose did not stimulate islet tissue respiration, this does not mean that glucose can not be utilized by islet tissue. There are a number of reasons why an added substrate might not stimulate respiration, such as its failure to readily enter the cell or the presence of sufficient endogenous substrate to saturate the enzyme system concerned. This is emphasized by our finding that

TABLE V

Effect of succinate on the respiration of toadfish islet and liver slices

Medium	Islet				Liver			
	Tonicity of medium (equiv. NaCl conc. *M*/liter)	No. of deter.	Ave. mμl. O_2/μg. prot./hr.	σ	Tonicity of medium (equiv. NaCl conc. *M*/liter)	No. of deter.	Ave. mμl. O_2/μg. prot./hr.	σ
0.054 *M* PO_4	0.0755	14	1.1	0.61	0.0755	9	2.9	0.37
0.054 *M* PO_4 + 0.0645 *M* NaCl	0.140	14	1.5	0.64	0.140	9	2.7	0.37
0.054 *M* PO_4 + 0.0645 *M* succinate	0.172	13	2.2	0.77	0.140*	9	4.9	0.89

* 0.043 *M* succinate used in this case.

the addition of glucose did not stimulate liver respiration, despite the known occurrence of the glycolytic system in the liver of most species. Our studies with fluoride and iodoacetate provide suggestive evidence that at least two enzymes of the glycolytic scheme (enolase and phosphoglyceraldehyde dehydrogenase) are also present in islet tissue. Neither of the inhibitors can be considered specific, but phosphoglyceraldehyde dehydrogenase is extremely sensitive to iodoacetate (Meyerhoff and Kiessling, 1933; Adler, Euler and Gunther, 1938) and at least one of the actions of fluoride is to inhibit enolase (Warburg and Christian, 1941).

Of the enzymes involved in the tricarboxylic acid cycle, the presence of α-ketoglutarate oxidase and succinoxidase are indicated by the stimulation of oxygen uptake observed following the addition of their respective substrates. The presence of succinoxidase is further indicated by the observed inhibition by malonate (43%). This inhibition can be compared with the 50–60% inhibition of rat liver homogenates reported by Holtkamp and Hill (1951) and Pardee and Potter (1949) as well as the 70% inhibition of rat brain homogenates (using 0.02 *M* malonate) reported by Pardee and Potter (1949). Finally, the participation of succinoxidase in islet tissue respiration is also consistent with the previous demonstration of the presence of succinate-cytochrome *c* reductase in this tissue (Lazarow and Cooperstein, 1951).

Neither pyruvate nor isocitrate stimulated islet respiration but this could be due to failure of these substrates to penetrate to their site of utilization. The

TABLE VI

Effect of isocitrate on the respiration of toadfish islet slices

Medium	Tonicity of medium (equiv. NaCl conc. *M*/liter)	No. of deter.	Ave. mμl. O_2/μg. prot./hr.	σ
0.054 *M* PO_4	0.0755	12	2.2	0.58
0.054 *M* PO_4 + 0.645 *M* NaCl	0.140	12	2.2	0.57
0.054 *M* PO_4 + 0.032 *M* isocitrate	0.140	12	2.3	0.45

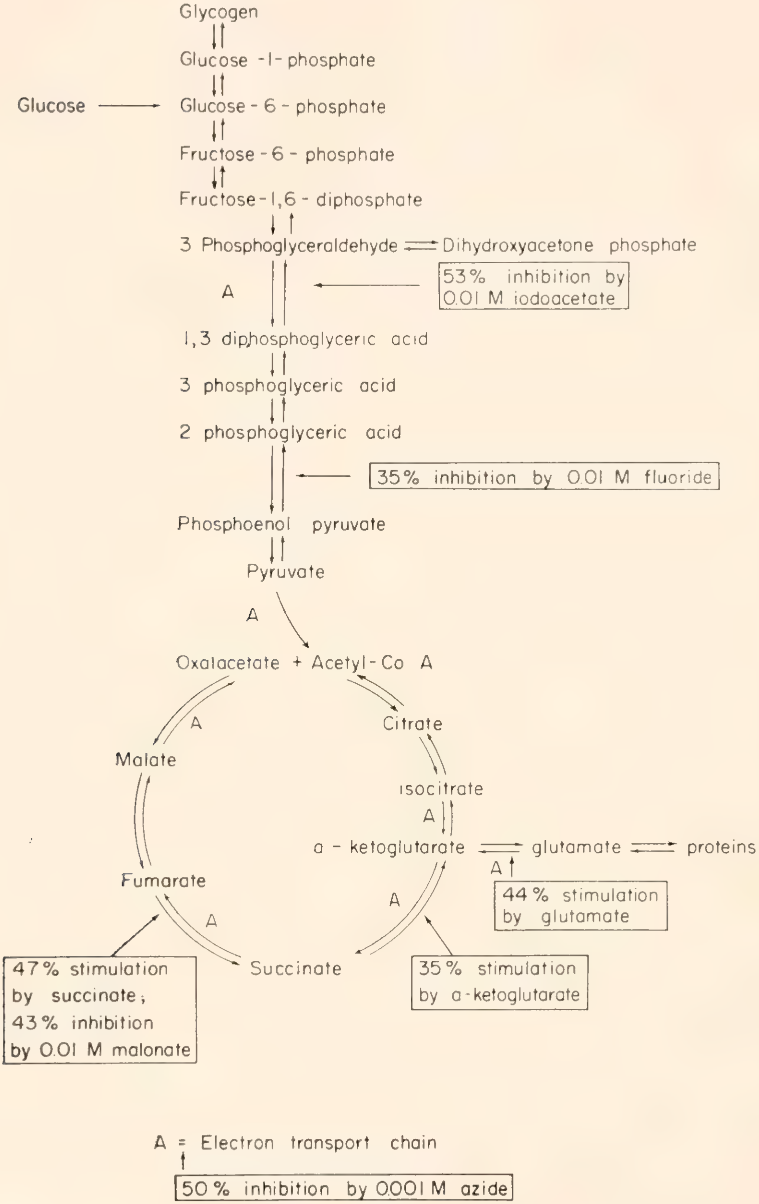
Glycogen
Glucose -1- phosphate
Glucose
Glucose - 6 - phosphate
Fructose - 6 - phosphate
Fructose - 1,6 - diphosphate
3 Phosphoglyceraldehyde
Dihydroxyacetone phosphate
A
53% inhibition by 0.01 M iodoacetate
1,3 diphosphoglyceric acid
3 phosphoglyceric acid
2 phosphoglyceric acid
35% inhibition by 0.01 M fluoride
Phosphoenol pyruvate
Pyruvate
A
Oxalacetate + Acetyl-Co A
A
Citrate
Malate
isocitrate
A
a - ketoglutarate
glutamate
proteins
A
44 % stimulation by glutamate
Fumarate
A
A
Succinate
47 % stimulation by succinate;
43 % inhibition by 0.01 M malonate
35 % stimulation by a-ketoglutarate
A = Electron transport chain
50 % inhibition by 0.001 M azide

FIGURE 1. A simplified scheme of carbohydrate metabolism, which indicates the evidence obtained for its operation in islet tissue metabolism.

TABLE VII

Effect of inhibitors on the respiration of toadfish islet tissue. (Each inhibitor was studied in a separate series of experiments and compared with controls run at the same time)

Medium	No. of deter.	Ave. mμl. O_2 μg. prot. hr.	σ	% inhibition
0.054 *M* PO_4	13	2.0	0.85	
0.054 *M* PO_4 + 0.01 *M* fluoride	14	1.3	0.37	35
0.054 *M* PO_4	16	1.9	0.74	
0.054 *M* PO_4 + 0.01 *M* iodoacetate	13	0.9	0.45	53
0.054 *M* PO_4	12	2.1	0.43	
0.054 *M* PO_4 + 0.01 *M* malonate	12	1.2	0.37	43
0.054 *M* PO_4	8	2.0	0.79	
0.054 *M* PO_4 + 0.001 *M* azide	8	1.0	0.24	50

enzymes of the tricarboxylic acid cycle are localized within the mitochondria, and it has been reported (Schneider, Striebich and Hogeboom, 1956) that the mitochondrial membrane may not be permeable to citrate.

The ability of islet to utilize glutamate is of considerable interest since this compound is a key link between the tricarboxylic acid cycle and protein synthesis.

The existence of cytochrome oxidase in islet tissue has been reported previously (Lazarow and Cooperstein, 1951) and its participation in the electron transport system is indicated by the inhibition of respiration produced by azide,[4] a known inhibitor of cytochrome oxidase (Keilin, 1936).

Figure 1 depicts a simplified scheme of carbohydrate metabolism, and indicates the evidence obtained for its operation in islet tissue metabolism.

SUMMARY AND CONCLUSIONS

The effects of substrates and inhibitors on the metabolic activity of toadfish islet slices were measured in the Cartesian diver microrespirometer under varying experimental conditions. Of the substrates added, α-ketoglutarate, glutamate, and succinate increased islet respiration while glucose, pyruvate, and isocitrate were ineffective. Iodoacetate, fluoride, malonate, and azide inhibited islet respiration.

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[4] Studies with cyanide have not been carried out to date because these are very difficult to perform in the Cartesian diver.

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A STUDY OF REPRODUCTION IN THE INTERTIDAL BARNACLE, MITELLA POLYMERUS, IN MONTEREY BAY, CALIFORNIA[1, 2]

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The goose barnacle, *Mitella polymerus* (Sowerby, 1833), sometimes referred to as *Pollicipes polymerus* or the leaf barnacle, is distributed along the exposed rocky coast of Western North America from British Columbia to the middle of Baja California (Cornwall, 1925). It is generally abundant here in the upper two-thirds of the intertidal belt (Ricketts and Calvin, 1952), though it is occasionally found below this level where there is considerable surging wave action. Along the central California coast, clusters of *Mitella* patch the exposed rocky regions, the barnacles usually attaching themselves to rock, to *Mytilus californianus*, or to other individuals of *Mitella*. Individuals are seldom isolated and one sees among the mussel beds or on the rocks rosette-shaped clusters in which large barnacles are at the center and smaller barnacles grade toward the periphery. In other aggregations, individuals of nearly the same size are packed closely together, frequently with such uniformity in size and orientation that their valves form a geometric pattern, and their closely packed bodies make a strong but resilient mat against the pounding surf. Often where the animals are attached to rock beneath the mussel beds, their stalks extend up eight inches or more to the surface. Occasionally, solitary animals occur on tables of rock exposed to strong wave action; in these the stalks remain short and stubby while the shells and bodies grow.

While *Mitella polymerus* is abundant, conspicuous, and well-known taxonomically, almost nothing is known of its reproductive biology. Nussbaum (1890) has described the anatomy of *M. polymerus* in some detail. The only published study of reproduction and development in this genus is that of Batham (1944–45), on the New Zealand species, *Mitella spinosus*. Since Batham's study of the reproductive cycle was carried out at latitude 44°52′ South, a comparison of her results with the situation occurring in *M. polymerus* at Monterey Bay (36°40′ North) seemed particularly interesting. The following study of reproduction in *M. polymerus* includes: the anatomy of the reproductive system, the relationship between size and sexual reproduction, the seasonal reproductive cycle, the rate of egg and embryo development, an estimate of fecundity, and evidence concerning self-fertilization.

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[2] The author wishes to express her gratitude to Dr. Donald P. Abbott, under whose guidance this work was carried out; and to acknowledge with appreciation his generous help with arrangement and discussion of the data.

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The Reproductive System

The gross reproductive anatomy of *Mitella polymerus* is shown in Figure 1. Ovarian tissue is found in the upper portion of the peduncle. From the ovaries, a pair of oviducts lead up into the body proper, emptying into the glandular oviducal atria in the bases of the first thoracic cirri. Eggs pass down the oviducts to the atria, where they receive a protective coating which glues them together into masses. The egg masses are then extruded through the oviducal apertures and come to lie in the mantle cavity on either side, where they are pressed flat to form the two ovigerous lamellae.

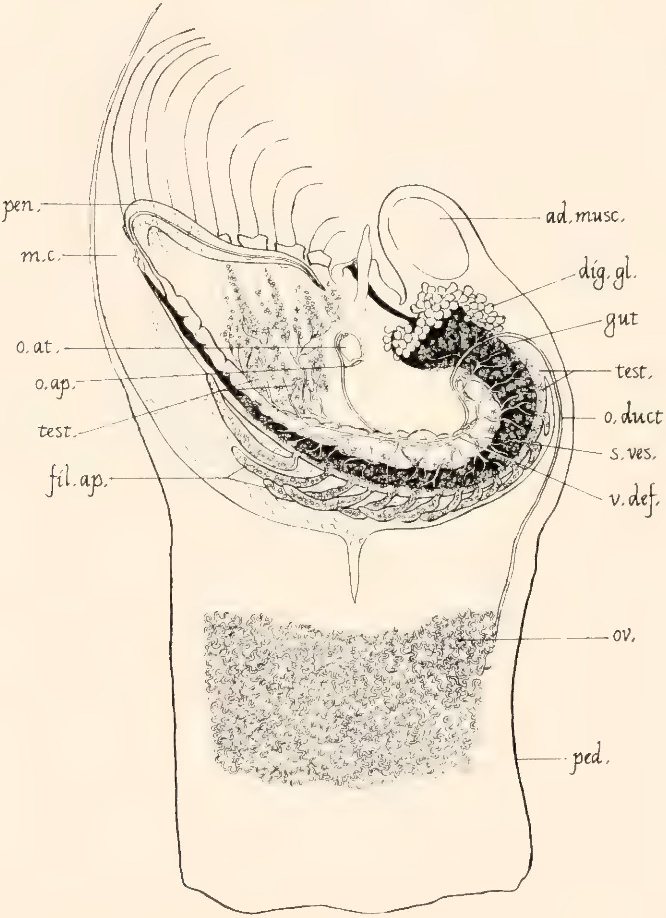

Figure 1. Gross anatomy of the reproductive system of *M. polymerus,* exposed by dissection from the left side. ad. musc. = adductor muscle; dig. gl. = digestive gland; fil. ap. = filamentary appendages; gut = gut; m. c. = mantle cavity; o. ap. = oviducal aperture; o. at. = oviducal atrium; o. duct = oviduct; ov. = ovaries; ped. = peduncle; pen. = penis; test. = testes; s. ves. = seminal vesicle; v. def. = vas deferens.

The numerous small testes are found on either side of the gut, extending ventrally into the coxopodites of the first four pairs of thoracic appendages and dorsally into the numerous paired filamentary appendages. Fine efferent ducts connect the testes to the paired vasa deferentia, which lead to the paired storage organs or seminal vesicles. These in turn join posteriorly at the base of the penis, and form a single duct extending to its tip.

Copulation was not observed, but sperm are deposited in the mantle cavity. The embryos are brooded in the ovigerous lamellae in the mantle cavity until they are hatched out as nauplius larvae.

The gross reproductive anatomy of *M. polymerus* is similar to that of *M. spinosus;* but *M. spinosus* has no filamentary appendages and the testis is described as (p. 370) "a median structure lying closely in the U-bend of the gut," with a pair of ducts leading from either side of it which expand to form the seminal vesicles (Batham, 1944–45).

MATERIALS AND METHODS FOR THE STUDY OF REPRODUCTION

Nearly all living material used in the present study was collected in or very near Monterey Bay, California, and studies were carried out at the Hopkins Marine Station of Stanford University, Pacific Grove.

For the study of the reproductive cycle, a population of *Mitella polymerus* was sampled at approximately monthly intervals for a period of fifteen months. All individuals were collected within an area approximately fifteen feet square on granite rocks and adjacent beds of *Mytilus californianus* on the northern shores of Mussel Point, Pacific Grove, California, at an intertidal level corresponding to the upper middle horizon of Zone Three of Ricketts and Calvin (1952). Care was taken to insure that all barnacles large enough to be reproductively mature were collected in close proximity to other individuals of a reproductive size, and to avoid taking isolated individuals which had lacked the opportunity for cross-fertilization.

Animals were anaesthetized for four hours in a solution of magnesium chloride isotonic with sea water. This was sufficient to relax the cirri and peduncle. The animals were then preserved in 10% formalin buffered with borax.

Individuals of all sizes were collected and examined. For most of the reproductive data, the largest common animals available were used. All of these were of a reproductive size, and for purposes of consistency, large animals which ranged in breadth (distance from rostrum to carina) from 27.5 mm. to 32.5 mm. were used (occasionally larger animals are found).

When maturing, the ovarian tissue in the peduncle (Fig. 1) undergoes marked and visible changes. Tiny eggs appear and grow, accumulating yolk until they are passed up through the oviducts and are extruded into the mantle cavity. Preliminary observations showed that throughout the ovarian tissue, eggs are generally of about the same size and stage of development at any one time; an exception to this is provided where a new batch of tiny eggs appears in the ovary before the previous batch of very much larger eggs is extruded. With a compound microscope and calibrated ocular micrometer, the greatest diameters of five to ten eggs were measured (in each animal), and an average ovarian egg size was recorded for the individual. Where two batches of eggs were present at once in an ovary (*i.e.,* large and small), this situation was quite evident, and the two were treated separately. The average egg sizes were then grouped into three useful classes for purposes of comparison: small eggs (diameter 0.016 mm. to 0.065 mm.), medium eggs (diameter 0.066 mm. to diameter 0.099 mm.), and large eggs (diameter 0.100 mm. to diameter 0.127 mm.). The few individuals in which no eggs could be seen were treated separately.

As the male gonads of *Mitella* mature, they also show easily visible changes: testes in the filamentary appendages and throughout the body lose their translucency and become opaque white with sperm. Sperm then travel through efferent tubules and vasa deferentia to the paired seminal vesicles, where they are stored. As more and more sperm accumulate in the seminal vesicles, these, too, change from translucent to opaque white and increase in diameter. Diameter of the seminal vesicles affords a fairly good index for the maturation of the male reproductive organs. Width was measured with a pair of dividers at a point just back of the anterior sharp bend in the seminal vesicle (Fig. 1). Conditions of the male organs were finally grouped into three categories: (1) seminal vesicles translucent and apparently empty of sperm; (2) seminal vesicles ranging in width from one to two mm.; and (3) seminal vesicles more than two mm. in width (ranging up to a maximum observed width of 3.8 mm.).

Fertilized eggs and developmental stages present in ovigerous lamellae were also studied. Embryos were examined in the ovigerous lamellae from animals taken in the monthly samples. In a pair of ovigerous lamellae taken from any one parent, embryos are all at about the same stage of development. As the fertilized egg develops, it increases slightly in size, but not enough for size to yield a good criterion for stage of development. Major morphological changes can be studied fairly readily, and it proved practicable for the purpose of the present problem to divide embryonic development into three stages: (1) early stages, with neither limb buds nor nauplius eye; (2) middle stages, with limb buds developing but no nauplius eye; and (3) late stages, with well-formed limbs and median eye present. Studies were also made of lamellae removed from the parent and raised *in vitro.* Ovigerous lamellae were isolated from their parent barnacles, placed in clear glass dishes of filtered sea water, and kept at a constant 13° C. in a water bath or a refrigerated room. Sea water was changed approximately daily, at which times the embryos were examined in small sections of the ovigerous lamellae plucked off with glass needles. Many embryos raised *in vitro* were observed through development to hatching, and many larvae were raised beyond this through several naupliar molts.

SIZE AND SEXUAL REPRODUCTION

Figure 2 shows the occurrence of ovigerous lamellae in animals of different sizes at selected times during the breeding season (May through December). Animals with a breadth of more than 27.5 mm. were always found to be sexually mature during the reproductive season; all individuals examined bore ovigerous lamellae, or enlarging ova in the ovaries, or both. In the individuals below 27.5 mm. breadth, the percentage of animals with ovigerous lamellae can be seen to decrease more or less directly with decreasing size. For individual months, the tendency is not always clear, and this may be due to sampling deficiencies (*e.g.,* class III in July and September). However, the summary graph, which represents values for the total number of animals of each size class, points out the trend well, and the differences shown here are statistically significant. The variation in reproductive activity with size may be explained in either of two ways: possibly a lower percentage of the smaller animals are sexually mature; or (less likely) the smaller class animals produce ovigerous lamellae less frequently than the larger animals. No ovigerous lamellae were ever found in any barnacle with a breadth of less than 17.2 mm.

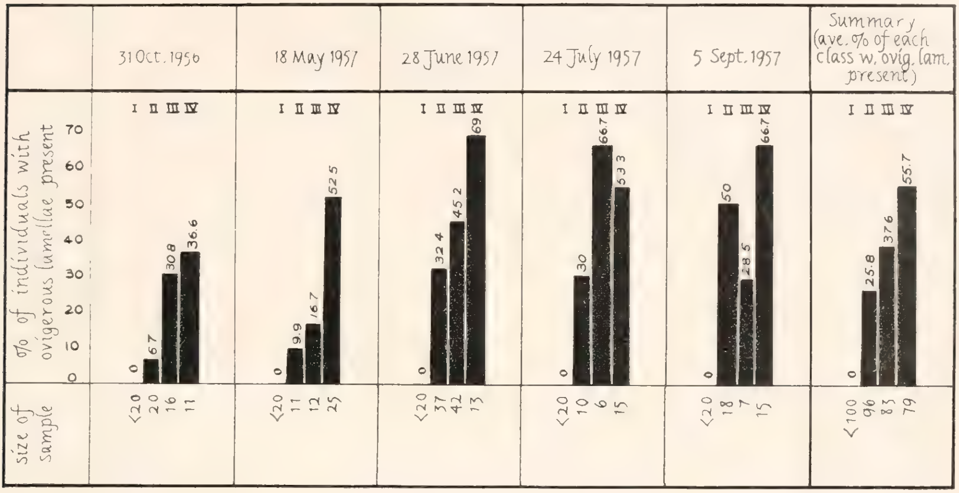

FIGURE 2. Variation in reproductive activity with size of animals. Vertical bars indicate percentage of individuals of each size class which contained ovigerous lamellae. Size classes are designated as follows: I, breadth less than 17.2 mm.; II, breadth 17.2 to 22.5 mm.; III, breadth 22.6 to 27.5 mm.; and IV, breadth 27.5 to 32.5 mm.

THE REPRODUCTIVE CYCLE IN THE MITELLA POLYMERUS POPULATION

Each month, 10 to 25 large animals were collected, measured, and examined for reproductive condition. The number of individuals carrying ovigerous lamellae was noted; conditions of the female and male gonads, and stage of development of the embryos (where present) were studied.

The distribution of egg sizes and larval stages is shown in Table I and Figure 3. Table I shows for each sample the number and per cent of *individuals* which contained given egg size classes and which brooded particular embryonic stages. However, in Figure 3, each separate mass of eggs or embryos is treated as a separate unit. For example, in cases where a given individual contained simultaneously small eggs, large eggs, and late embryos, these appear in Figure 3 in three different horizons in the same date column.

It is apparent in Figure 3 that between November, 1956, and January, 1957, breeding waned and was discontinued until the spring of 1957. Then, during March, eggs began to enlarge, but did not exceed the upper limits of the class of small eggs. By April, the first medium and large ovarian eggs were present, and the first fertilized egg mass appeared. Egg production continued through the year to January, 1958, when the number of ovigerous lamellae present in the population dropped significantly. The season of reproductive activity for the population covers three-quarters of the year, and it is of interest that all stages of egg and embryo development were found throughout the season. On the average, during the height of the breeding season, between 50% and 60% of the population of large animals are carrying ovigerous lamellae at any one time.

Data for the male gonads are plotted in Table I and in Figure 4. Sperm is present in at least some members of the population throughout the year. While there is seasonal variation in the condition of the male gonads, this is less well defined than is that of the female gonads. In the fall of 1956 and through January, 1957, some seminal vesicles were very thick with sperm, while others appeared spent. In February and March, 1957, most of the vesicles had a meager amount of sperm, presumably building up, and some were empty. In April, most vesicles were quite full with sperm, others had a meager amount, and none were empty, and from this point on through the summer and fall of 1957, the mean vesicle width was high (well over two mm.). Empty vesicles were not observed again until January, 1958. Thus during the winter months, the quantity of sperm present in the seminal vesicles of most of the population was considerably less than the amount present during the rest of the year.

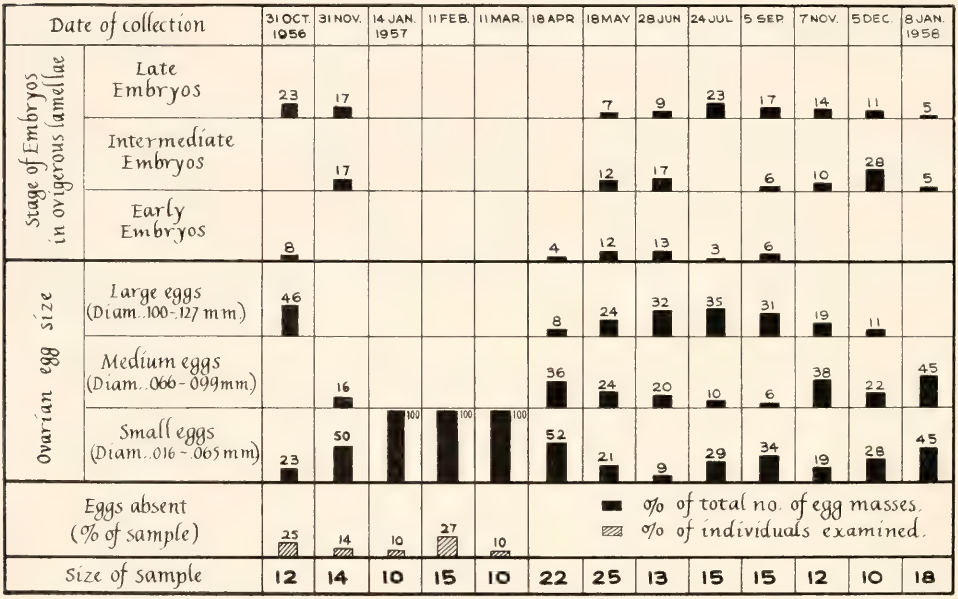

FIGURE 3. Distribution of egg and embryo masses of various stages of development in large *M. polymerus.*

There is a suggestion of waves of reproductive activity during the breeding season, in the data presented in Figure 3 and Table I. This appears most clearly in Table I in the column showing the percentage of animals which are carrying

TABLE I

Seasonal variation in egg size, seminal vesicle width, and stage of development of brooded embryos in M. polymerus

Dates	1956 Oct. 31	Nov. 30	1957 Jan. 19	Feb. 11	Mar. 11	Apr. 18	May 18	Jun. 28	Jul. 24	Sept. 5	Nov. 7	Dec. 5	1958 Jan. 8
No. of animals examined:	12	14	10	15	10	22	25	13	15	15	12	10	18
No. and per cent of animals with:													
Eggs absent													
No.	3	2	1	4	1	—	—	—	—	—	—		—
%	25	14	10	27	10	—	—	—		—		—	
Small eggs													
No.	3	9	9	11	9	11	5	1	1	2	—	4	9
%	25	65	90	73	90	50	20	8	7	13	—	40	50
Medium eggs													
No.	—	3	—	—	—	9	10	6	3	2	8	4	9
%	—	21	—	—	—	41	40	46	20	13	67	40	50
Large eggs													
No.	6	—	—	—	—	—	6	5	3	1	—	1	—
%	50	—		—	—		24	38	20	7	—	10	—
Both small and Large eggs													
No.	—	—	—	—	—	2	4	1	8	10	4	1	
%	—	—	—	—	—	9	16	8	53	67	33	10	—
No. and per cent of animals with ovigerous lamellae:													
No.	4	6	—	—	—	1	13	9	8	10	5	7	2
%	33	43	—	—	—	5	52	69	53	67	42	70	11
No. and per cent of animals with ovigerous lamellae bearing:													
Early embryos													
No.	1	—	—	—	—	1	5	3	1	2	—	—	—
%	8	—	—	—	—	5	20	23	7	13.5	—	—	—
Middle embryos													
No.	—	3	—	—	—	—	5	4	—	2	2	5	1
%		21.5	—	—	—	—	20	31	—	13.5	17	50	5.5
Late embryos													
No.	3	3	—	—	—	—	3	2	7	6	3	2	1
%	25	21.5		—	—	—	12	15	46	40	25	20	5.5
Conditions of seminal vesicles present in the population													
No. of animals examined:	12	13	18	15	10	21	23	13	14	14	12	10	18
No. of animals with seminal vesicles in following conditions:													
Apparently empty	1	1	1	4	3	—	—	—	—	—	—	—	2
Width 1–2 mm.	5	6	5	9	7	4	1	2	2	5	4	9	7
Width 2–3.8 mm.	6	6	12	2	—	17	22	11	12	9	8	1	9

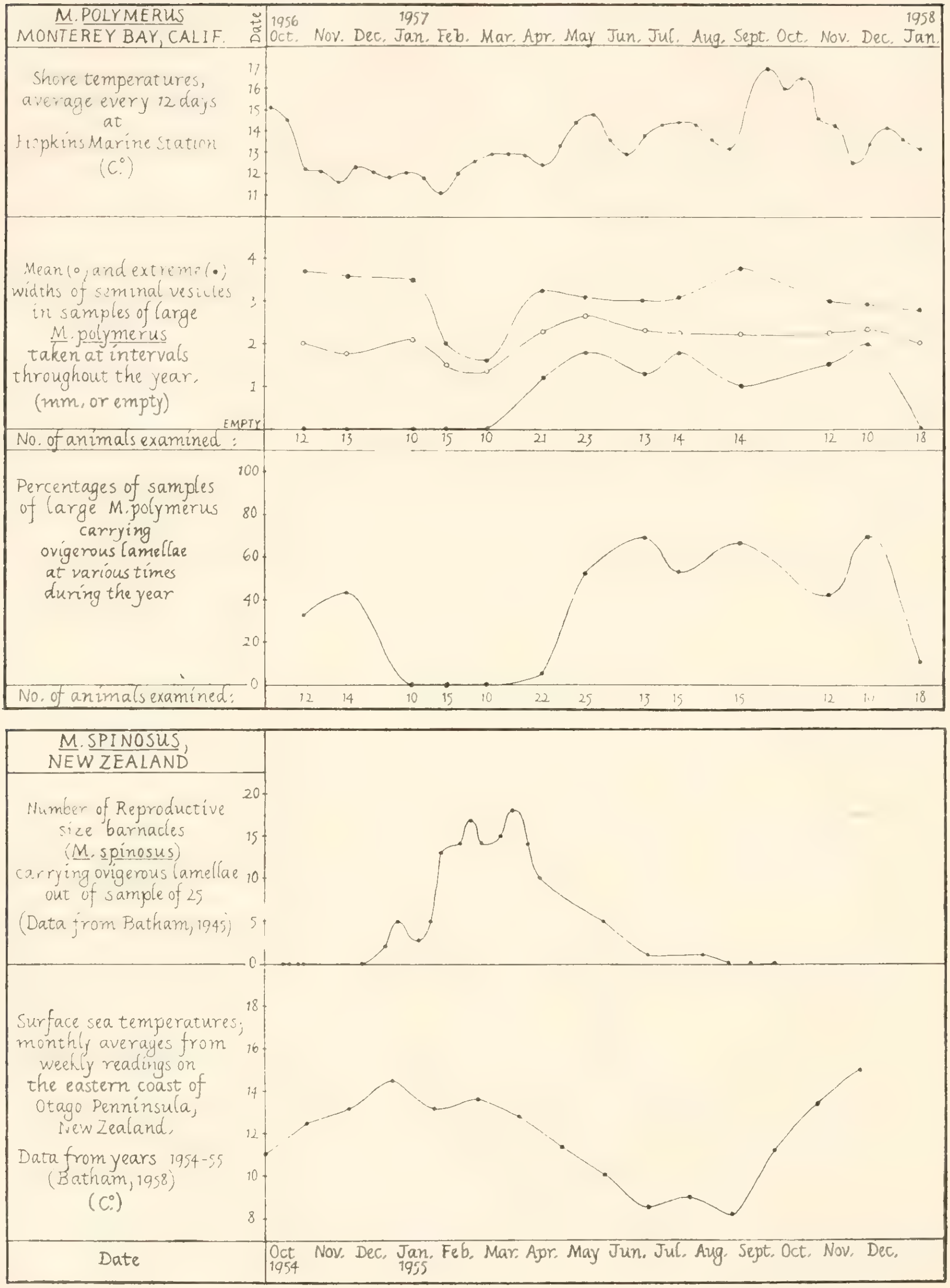

FIGURE 4. Temperature and reproductive activity in *M. polymerus* in Monterey Bay and in *M. spinosus* in New Zealand.

ovigerous lamellae at different times during the year. The highest values, *i.e.*, those for June, September, and December (69%, 67%, and 70%, respectively), alternate with the lower values shown for May, July, and November (53%, 53%, and 42%, respectively), and therefore suggest that the population as a whole may be reproducing in waves, more or less simultaneously. As will be indicated later, this phenomenon is very likely related to the successive waves of reproductive activity shown by *individual* barnacles during the breeding season. However, not all individuals start to reproduce at precisely the same time, and waves of activity do not proceed in all individuals at exactly the same rate. The differences between alternate highs and lows and their deviations from the mean for the population are not statistically significant; and a larger sample might be expected to yield a smoother plateau for reproductive activity of the population.

The Reproductive Cycle in Individuals

The reproductive cycle in the population of *M. polymerus* represents a summation of the reproductive processes in individual barnacles. The animals are hermaphroditic and ovoviviparous, brooding their young for a period of time in the mantle cavity. It is accordingly of interest to examine the relative degrees of development of the two gonads in individual animals, and to relate the ovarian egg size with the stage of development of brooded embryos present simultaneously in individual barnacles.

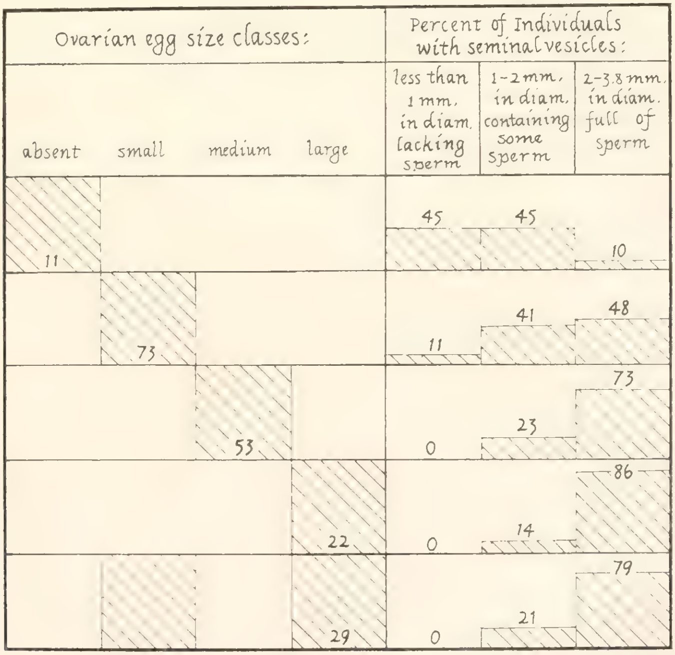

FIGURE 5. Egg sizes and seminal vesicle widths found simultaneously in large individuals. Left-hand side: numbers within the blocks represent numbers of individuals examined; right-hand side: numbers above blocks represent percentages of individuals with seminal vesicles of given sizes.

For studies of the two gonads, the large animals taken at intervals throughout the study period were examined. Animals were grouped into four categories according to egg size: animals with small eggs, those with medium eggs, those with large eggs, and those with both small and large eggs. The seminal vesicle width of each animal of a given egg category was noted. The data for all animals examined are shown in Figure 5.

In Figure 5, the bars on the right hand side represent the frequency of occurrence of the various conditions of the seminal vesicle which may be found in individuals with eggs of any given size category. We see from the graph that sperm is absent only in animals where eggs are small or absent. Under all other ovarian conditions, sperm is always present, and in the majority of barnacles with medium or large eggs, the seminal vesicles are full.

TABLE II

Correlation of size of ovarian egg with developmental stage of brooded embryos present in the same individual. Numbers of individuals showing each combination of conditions are indicated by numbers within the squares. Heavy vertical lines enclose the "peak" months of the breeding season.

Egg size	Embryos	31 Oct.	30 Nov.	14 Jan.	11 Feb.	11 Mar.	18 Apr.	18 May	28 June	24 July	5 Sept.	7 Nov.	5 Dec.	8 Jan.
Small & Large	Absent						2	4	1	4	6	3	1	
	Early													
	Middle													
	Late									5	4	1		
Small	Absent	2	5	9	11	9	11			1				8
	Early							4	1		1			
	Middle		2					1			1		3	
	Late	1	2										1	1
Medium	Absent		1				9	3				4	1	8
	Early							1	1	1				
	Middle		1					4	4		1	2	2	1
	Late		1					2	1	1	1	2	1	
Large	Absent	4						5	4	2			1	
	Early													
	Middle													
	Late	2						1	1	1	1			
Eggs Absent	Absent	3	2	1	3	1								
	Early													
	Middle													
	Late													
		12	14	10	14	10	22	25	13	15	15	12	10	18

Table II contains data illustrating the conditions which may occur simultaneously in the ovary and in the brooded ovigerous lamellae, in the same individual at different times of the year. From these data, the information on large animals collected during the breeding season (October to November, 1956; and May, 1957 to January, 1958) and containing both ovarian eggs and ovigerous lamellae, has been selected and is shown graphically in Figure 6.

It is evident from Figure 6 that there is a general relationship between ovarian egg size and brooded embryo stage in the same individual, particularly during the height of the breeding season. In general, where eggs are small in the ovaries, embryos are in an early stage of development in the mantle cavity; where eggs are medium-sized in the ovaries, embryos are in the middle stage of development in the mantle cavity; and where eggs in the ovaries are large (nearly ready to be extruded), embryos in the mantle cavity are advanced (nearly ready to be liberated). The parallel diagonal lines drawn through successive peak conditions show clearly the synchronized pattern of development of ovaries and embryos during the peak months of the reproductive season. Certainly such a condition, in which eggs and embryos show more or less parallel development, appears to represent the most efficient timing pattern for an ovoviviparous organism which produces successive broods of young in a single breeding season. However, it is clear from the graph and the table that precise coordination between rates of egg enlargement and larval development does not exist for all animals examined during the reproductive season. For example, some animals with small eggs in the ovaries were found brooding middle or late stage embryos. In such cases, it appears that the interval between successive broods of larvae is greater than in cases where eggs and embryos develop in phase, and that ovarian egg development is retarded with respect to embryonic development. Table II shows clearly that these animals with relatively retarded ovarian development tend to be localized in time, predominating during the later months and not the peak months of the reproductive season. Thus, the five cases in which late embryos accompany small

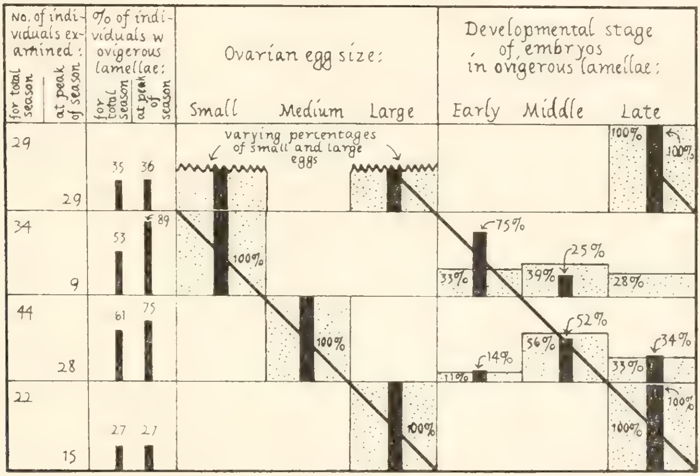

FIGURE 6. Developmental stages, within individuals, of ovarian eggs and brooded embryos, where both are present simultaneously. Numbers pointing to black bars show percentages of given stages present during the "peak" months of the reproductive season; numbers within the larger bars show percentages of given stages present during the total reproductive season.

ovarian eggs occurred in October and November of 1956, December of 1957, and January of 1958, suggesting that at this time development of the ovaries had slowed down or stopped for the season, and that the final batch of nauplii would soon have been shed. Also, the combination of small eggs with middle embryonic stages occurred, with one exception, either at the beginning (May, 1957) or near the end (November, 1956 and December, 1957) of the breeding season. Animals with medium eggs and *no* ovigerous lamellae occurred in April and May, 1957 (when they were presumably giving rise to their first batch of eggs of the season) and in November, 1956 and 1957 (when they may represent individuals in which the ovarian growth has slowed down toward the close of the season). In contrast, a few animals contained medium-sized eggs along with early embryos in the ovigerous lamellae, suggesting a relative acceleration of egg development. However, in all three cases, the eggs measured fell close to the lower size limits of the medium egg size class, and the apparent acceleration is exaggerated by the positioning of size-class limits in the grouping of data.

TABLE III

Apparent conditions of ovaries (ovarian eggs) with relation to embryos (found simultaneously in an individual)

Dates	Number of times ovaries appeared in following conditions:		
	Accelerated	Synchronized	Retarded
31 Oct. 1956		2	1
30 Nov. 1956		1	5
14 Jan. 1957		no reproductive activity	
11 Feb. 1957			
11 Mar. 1957			
18 Apr. 1957			
18 May 1957	1	9	3
28 Jun. 1957	1	6	1
24 Jul. 1957	1	6	1
5 Sept. 1957		7	2
7 Nov. 1957		3	2
5 Dec. 1957		2	5
8 Jan. 1958		1	1

Table III summarizes the condition of the ovaries with relation to embryonic stages. We see that during the early and middle months of the reproductive season, most of the animals examined appeared to show synchrony in rates of ovarian and embryonic development. Toward the end of the reproductive season, a relative lag in ovarian egg development became noticeable. By combining data for the months of May through September (the main reproductive period) and for the period of October through December (the end of the breeding season), we can show that the prevalence of animals with synchronized ovaries during the former months, and of animals with retarded ovaries during the latter months, is indeed statistically significant.

While animals with apparently well synchronized brood development are probably producing batches of eggs at a relatively efficient rate, there is evidence that even in such animals some time does elapse between broods. We might expect, in a perfectly synchronized animal, that immediately following the liberation of a batch of larvae, a second batch of eggs would be extruded, and that such an animal would be carrying ovigerous lamellae virtually all of the time. However, at no time during the reproductive period were all reproductively mature members of the population carrying ovigerous lamellae; even during the height of the breeding season, only 50% to 60% of the population were brooding embryos. The occurrence of a very few slow developers during this time could hardly account for such a discrepancy; and it must be assumed that even during the height of reproduction, some time does elapse between the hatching of a batch of larvae and the extrusion of the next batch of eggs from the same individual.

NUMBER OF BROODS AND LARVAE LIBERATED BY MITELLA POLYMERUS, AND ITS RELATIVE FECUNDITY

No direct observations are available on the number of broods produced by an adult barnacle per year. However, by combining all the lines of evidence at hand, we can arrive at a hypothetical figure for the number of batches of larvae liberated by a large individual during one reproductive season. Three lines of evidence are considered here: the duration of the reproductive season, the rate of development of embryos in the ovigerous lamellae, and the developmental stages of eggs and embryos present at any one time in an individual during the reproductive season.

We have already seen that the reproductive period for most barnacles extends through about eight months or about 240 days (Fig. 3). The approximate duration of the lamellar brood period was determined in the laboratory. Several batches of larvae were raised *in vitro* at 13° C., starting with what appeared to be the two-cell stage, and continuing to hatching. Periodic examination of these ovigerous lamellae showed that embryos remain in the "early" stage for the first nine days, are in the "middle" stage from the tenth through the fifteenth day, and remain in the "late" stage from the sixteenth day to hatching, which occurs on the twenty-ninth to the thirty-first day. Development from fertilization to hatching of the nauplius thus averages about thirty days. This rate is substantiated for field conditions in Figure 3, where we find the first large eggs being extruded in April and the first late embryo just one month later. It is interesting to note that the brood period for "normal" *M. spinosus*, raised under conditions where temperature was not controlled but averaged 14° C. to 15° C., was thirty to thirty-two days (Batham, 1946).

The developmental stages present simultaneously in individuals have been shown in Figure 6 and Table II. Here, the simultaneous presence in single animals of three stages (small eggs, large eggs, and late embryos, especially in animals taken in July and September) strongly suggests that an animal may give rise to *at least* three batches of embryos during one reproductive season. Tables II and III show that during the first six months of the reproductive season, egg and embryo stages tended to be more or less synchronized in individuals; and during the last two months of the breeding season, the majority of animals carried broods which tended to be out of phase by two stages. In a perfectly synchronized animal, in which ovarian eggs and lamellar embryos develop in phase, we might assume that the interval between the average small egg and the average early embryo is equal to the lamellar brood period, or thirty days. In animals one step out of phase, that is, in animals in which *medium* embryos accompany *small* eggs, or *late* embryos accompany *medium* eggs, we might assume that the interval between broods is thirty-eight days (or thirty days plus the difference between the average age of an early embryo and the average age of a middle stage embryo—eight days). In animals two steps out of phase, in which *late* embryos accompany *small* eggs, or (specifically in November, 1957) *no* embryos accompany *medium* eggs, we might assume that the interval between successive broods is at least 48.5 days (or thirty days plus the difference between the average age of an early embryo and the average age of a late embryo—18.5 days).

Through the breeding season (eight months), these various intervals were found to be more or less localized in time. That is, for approximately the first six months, most of the population seemed to be producing broods either in phase or one stage out of phase. Hence, at the assumed rate of one brood for every thirty to thirty-eight days, a minimum of 4.7 broods and a maximum of six broods could have been extruded by an individual during this time. For the last two reproductive months, a majority of the population carried broods two steps out of phase. At the assumed rate, then, of one brood for every 48.5 days, one to 1.2 broods may have been extruded by an individual during these two months. During the TOTAL reproductive period, then, it appears that a single large barnacle *could* have liberated from five to 7.2 broods of larvae.

If an average animal gives rise to six broods in a season, each brood developing in the mantle cavity for thirty days, we would expect such an animal to be carrying embryos for approximately 180 days out of the total 240 days, or approximately 75% of the total time. Since no single individual was followed during the breeding season, no data are available to provide a direct means of checking this figure. However, 59% of the large individuals collected during the eight-month reproductive season bore ovigerous lamellae. This suggests that perhaps large individuals contain ovigerous lamellae for only about 59% of the total reproductive period, or 142 out of 240 days. On this basis, then, the average animal probably produced only four or five broods during the season.

There seem to be two good reasons for the discrepancy between the two estimates for average number of broods per season. The first, already mentioned, is that even in well-synchronized animals, some time did elapse between successive lamellar broods. There was no direct measurement of the duration of this minimum interval, and thirty days was used as the minimum time for the enlargement and extrusion of eggs; but actually, the period during which eggs remain in the ovary is probably a few days longer. A suggestion providing some independent support for this is seen in the data in Figure 3, where on March 11, 1957, all ovarian eggs were in the small size class (though some approached the upper limits of this class), and on April 18, 1957, 36 days later, one individual was just extruding eggs from the oviduct. The other possible reason for the discrepancy between the two estimates of number of broods produced per year is shown by the fact that many stages of development were found in the population at any one time, and that the whole population did not produce broods synchronously. It is probable that the average animal did not reproduce at the given rates for a total of 240 days; that animals may have either started reproducing later than the earliest assumed limit or stopped reproducing earlier than the latest assumed limit. Such animals may have produced only five or four, or possibly only three broods during a season. In contrast, the results of Batham (1944–45) show that *M. spinosus* in New Zealand probably liberates only two per season.

Counts have been made of the number of larvae contained in the ovigerous lamellae of both species of *Mitella*. It was found that a large *M. polymerus* may liberate from 104,000 to 240,000 larvae from a single brood contained in one pair of ovigerous lamellae; the slightly smaller *M. spinosus* liberates approximately 3000 larvae per brood (Batham, 1944–45). It appears that a single adult *M. polymerus* may produce roughly 52 to 280 times as many larvae per year as a single adult *M. spinosus*.

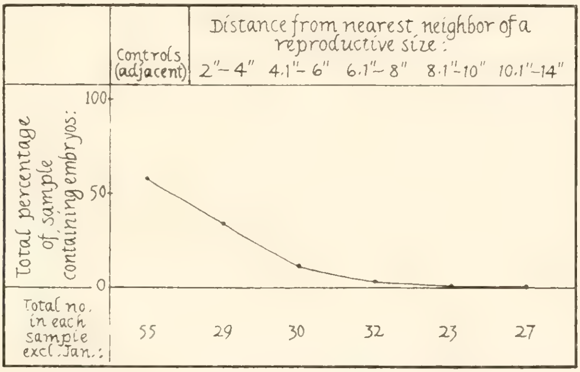

FIGURE 7. Summary graph showing percentages of large *M. polymerus* containing ovigerous lamellae when separated from other large specimens by given distances.

SELF-FERTILIZATION

For a study of self-fertilization, a series of large and relatively isolated barnacles, situated at various measured distances from their nearest neighbors of reproductive size, were collected and inspected for the presence of ovigerous lamellae. These samples were collected at different times throughout the reproductive season. The data, grouped for the months of collection, and for the distances by which the sexually mature barnacles were separated, are presented in Table IV and Figure 7. It can be seen that over 50% of the control animals for each month (except January, 1960) carried ovigerous lamellae; animals separated by two to four inches appeared to carry ovigerous lamellae less frequently, but the differences are not statistically significant. There is a statistically significant drop in the presence of embryos in animals separated from their nearest neighbor of a reproductive size by over four inches, and an absence of embryos in animals separated by more than eight inches. Thus it appears that eight inches was the maximum distance over which copulation of large animals collected could take place, and that animals separated by greater distances failed to receive sperm. Despite the

Table IV

Numbers of barnacles of reproductive size isolated from other such barnacles by given distances, showing presence (+) and absence (−) of ovigerous lamellae

Date	Distance from nearest neighbor of reproductive size:											
	Controls (adjacent)		2″–4″		4″–6″		6″–8″		8″–10″		10″–14″	
	+	−	+	−	+	−	+	−	+	−	+	−
1958	12	8	3	2	1	3	0	5	0	2	0	8
1959												
20 July	10	5	3	3	1	6	1	6	0	8	0	3
7 Aug.	6	4	2	8	0	10	0	12	0	7	0	6
2 Nov.	4	6	2	6	1	8	0	8	0	6	0	10
1960												
2 Jan.	0	30	0	8	0	6	0	5	0	8	—	—
Summary, excluding Jan. 1960	32	23	10	19	3	27	1	31	0	23	0	27

simultaneous maturity of male and female gonads, and despite the evidence for self-fertilization in other barnacle species (Barnes and Crisp, 1956), self-fertilization apparently does not take place in *M. polymerus.* It also appears that formation of ovigerous lamellae does not occur in the absence of fertilization.

Discussion

On the subject of reproduction in cirripeds, a good deal has been written. Some accounts, such as those of Darwin (1851), Broch (1922), and others are concerned with the questions of hermaphroditism, the existence of complemental males, etc., rather than reproductive anatomy, reproductive cycles, and fecundity.

Comparison of the seasonal reproductive cycle of *M. polymerus* in Monterey Bay with that of *M. spinosus* on the New Zealand coast (Batham, 1944–45) shows some interesting features. Figure 4 summarizes reproductive and temperature data for the two species. In Monterey Bay, from November, 1956, through the greater part of April, 1957, shore temperatures remained between 11.2° C. and 12.4° C., but showed a definite rise toward 13° C. during February and March. From May, 1957, through about one-half of November, shore temperatures remained above 13° C., rising to over 14° C. in May, July, and August, and to above 16° C. during September and October. Thus, the year may be roughly divided into the colder winter months and the warmer spring, summer, and fall months.

In *M. polymerus,* the increase in mean seminal vesicle width during the early spring roughly parallels the rise in shore temperature; the decrease in mean seminal vesicle width in the winter follows roughly the winter decrease in temperature. Likewise, the earliest occurrence of embryos follows closely behind the seasonal rise in temperature, and embryos continue to be present in the population until just after temperatures begin to drop during the winter. There is thus a clear correspondence between reproduction and shore temperature. The three peaks seen in the occurrence of embryos in the latter half of 1957, while not statistically significant, show a relationship with the temperature peaks of May, July, and September which is perhaps suggestive.

The data given by Batham (1944–45) for *M. spinosus*, from the open coast of Otago Peninsula, New Zealand, and the corresponding temperature curve (from two later years and a neighboring vicinity; Batham, 1958) show a similar relationship between temperature and reproductive activity. As might be expected for a related species occurring at a slightly higher latitude but in the southern hemisphere, the reproductive cycle is a rough mirror image of that of *M. polymerus* in Monterey Bay, California, though the breeding season is somewhat shorter.

Other barnacle species have been observed to be reproductively active primarily during the months of warmer water temperatures: *Balanus crenatus*, studied on the Atlantic coast of Canada (Bousfield, 1952–53) and in San Francisco Bay, California (Herz, 1933); *Balanus improvisus*, studied on the Atlantic coast of Canada (Bousfield, 1952–53); and *Chthamalus stellatus*, studied in Great Britain (Crisp, 1950).

While such a correlation between temperature and reproductive activity might seem obvious and only reasonable, a number of barnacles reproduce primarily when water temperatures are low or at a minimum. This group includes the following: *Balanus balanoides*, studied on the Atlantic coast of Canada (Bousfield, 1952–53), at Woods Hole, Massachusetts (Fish, 1925), and in Great Britain (Moore, 1935; Crisp and Patel, 1960); *Balanus hameri*, studied on the Atlantic coast of Canada (Bousfield, 1952–53), and in Great Britain (Crisp, 1954); *Balanus porcatus*, studied in Great Britain (Crisp, 1954); and *Balanus glandula*, studied at Vancouver, B. C., and La Jolla, California (Barnes and Barnes, 1956) and in San Francisco Bay, California (Herz, 1933).

There is evidence that still another group of barnacles reproduce (perhaps with some variation in rate) throughout the entire year. These include *Elminius modestus*, studied in Great Britain (Crisp and Davies, 1955), *Verruca stroemia*, studied in Great Britain (Pyefinch, 1948), and *Balanus tintinabulum*, observed at La Jolla, California (Coe, 1932).

Bousfield (1952–53) has studied the distribution and spawning seasons of the barnacles of the Atlantic coast of Canada, and reviewed the evidence supporting temperature as a principal factor governing reproduction in cirripeds. He clearly indicated that there is variability in reproductive period within a given species at different latitudes within its geographic range, and showed that reproductive activity tended to occur at times when temperatures were similar, regardless of latitude.

The relationship of temperature and food supply to rate of reproduction has been studied by Crisp and Davies (1955) in the barnacle *Elminius modestus*. By growing these barnacles on glass slides and observing development through the translucent bases, these workers were able to follow, *in vivo*, the development of both ovarian eggs and lamellar embryos. They found that (p. 379) "the time interval occupied by successive broods varies among individuals, and with the season of the year. Rate of development of embryos seems to be a function of temperature alone, but regeneration of the ovary depends on nutrition and food supply." Crisp (1959), working with *Balanus balanoides*, showed that the rates of development of the early embryonic stages (through the limb bud stage) are temperature-dependent up to 12°–14° C., but that the later stages vary little in rate of development between 3° C. and 12° C.

Further points of comparison may be brought out between *M. polymerus*, *M. spinosus*, and other barnacles. The present study indicated that sexual maturity is not necessarily a function of size of the animals alone. Results of studies on *M. spinosus* and *Elminius modestus* showed similarly that in populations of smaller barnacles, sexually mature individuals are found, but less frequently than in populations of larger barnacles.

Self-fertilization apparently does not occur in large isolated individuals of *M. polymerus*, and cross-fertilization appears to be the rule. Crisp (1954) and Crisp and Patel (1960) pointed out that cross-fertilization also appears obligatory in *B. crenatus*, *Elminius modestus*, and in *B. balanoides*. However, self-fertilization very probably can occur in at least three species of acorn barnacles. Barnes and Crisp (1956) experimentally isolated individuals of *Chthamalus stellatus*, *Verruca stroemia*, and *Balanus perforatus* and found that they frequently produced ovigerous lamellae. They also observed that fertilized eggs found in such isolated individuals are frequently less viable than cross-fertilized eggs. The genetic advantage of cross-fertilization is well known, and it appears that the *Mitella polymerus* population, composed usually of closely-packed individuals, is well adapted for cross-fertilization.

SUMMARY

1. The gross structure of the reproductive system of *M. polymerus* is described and compared with that of the southern hemisphere species, *M. spinosus* (studied by Batham, 1944–45).

2. Size and reproductive activity in *M. polymerus* are related. All animals over 27.5 mm. in breadth (distance from rostrum to carina) are found to reproduce; smaller animals are found to contain developing embryos less frequently. No sexually mature animals less than 17.2 mm. in breadth were found.

3. A fifteen-month study of the reproductive cycle in the population is described. Reproductive activity is evident during the greater part of the year. For the year of 1957, developing embryos were present in the population for a period of eight months during which time the shore temperature ranged from 12.3° C. to 17° C. The reproductive season for the southern hemisphere species likewise occurs during the warmest months; thus the yearly cycle of *M. polymerus* shows a perhaps expected mirror image of the situation occurring in the southern hemisphere.

4. Within individuals, male and female gonads mature at approximately the same time during the year, and during the greater part of the year, an individual contains both developing eggs and seminal vesicles full of sperm.

5. Stages of development of ovarian eggs and brooded embryos found simultaneously in individuals are compared. During the early and middle months of the reproductive season, ovarian eggs and brooded embryos tend to be in similar stages of development (that is, small eggs are found with early stage embryos, large eggs with late stage embryos, etc.). During the later reproductive months, a relative lag in ovarian egg development is evident.

6. Embryos were raised *in vitro* under controlled temperatures. The embryos took an average time of thirty days for development from fertilized egg to free-swimming larva.

7. Estimates are given of the number of broods of young and the number of larvae liberated by a large individual during a year, and these are compared with the results of Batham (1944–45) for *M. spinosus*. Studies of the larval brood period, the stages of eggs and embryos found simultaneously in individuals, and the length of the reproductive season allow an hypothesis that five to seven broods may be liberated by a large individual during a year. Three to four broods appears more probable for an average large animal. A pair of ovigerous lamellae may contain from 104,000 to 240,000 larvae. Batham's data (1944–45) showed that probably two broods, each containing approximately 3000 larvae, are liberated by *M. spinosus* during a year. Thus *M. polymerus* may liberate from 52 to 280 times as many larvae per year as a single large *M. spinosus*.

8. The possibility of self-fertilization in *M. polymerus* is studied. Relatively isolated large animals are found to carry ovigerous lamellae less frequently than those adjacent to each other; and large animals isolated from each other by over eight inches were never found carrying embryos. From this evidence, it appears that self-fertilization does not occur in this species, and that cross-fertilization is necessary for the formation of ovigerous lamellae.

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OBSERVATIONS ON THE NUTRITION OF THE RHYNCHOCOELAN LINEUS RUBER (O. F. MÜLLER)

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Feeding and digestion in the Rhynchocoela have received relatively little attention apart from brief accounts by Wilson (1900), Reisinger (1926), Coe (1943) and Hyman (1951). These indicate that the group is carnivorous, preying upon a variety of invertebrates which are captured by means of the extensible proboscis and swallowed whole, and that digestion may be either extracellular or partially intracellular. No further details of rhynchocoel nutrition are available and to remedy this deficiency, that of the common British species *Lineus ruber* (O. F. Müller) has been investigated.

Materials and Methods

Specimens of *Lineus ruber* were collected from beneath rocks embedded in sand at mid-tide level at Cremyll, Plymouth. After starvation for two days to induce a readiness to feed and to clear the gut of remnants of previous meals, individuals were presented with representatives of the fauna of their habitat, and the methods of capture and ingestion of the selected prey observed.

The course of digestion was followed by histological examination of individuals fixed at progressive intervals after feeding upon either the natural food or easily identifiable test foods such as frog erythrocytes and raw, optically active starch grains. Fixation was in Susa at 30° C. and sections cut at $8\,\mu$ were stained with the haematoxylin and eosin, Feulgen, periodic acid-Schiff (P.A.S.), Alcian blue (for mucin), benzidine-peroxide (for haemoglobin), and Lugol's iodine techniques. Changes in the pH of the gut contents during digestion were followed by feeding particles of fish muscle stained with 0.5% sea water solutions of various indicators and observing subsequent color changes by periodically flattening the fed individuals and examining by both reflected and transmitted light.

Food reserves were studied after fixation in Flemming (for fats) and 90% alcohol containing 1% picric acid (for carbohydrates and proteins). Sections of individuals fixed in the latter reagent were stained by the Best's carmine, P.A.S. and modified Millon's methods.

Observations

The food and feeding mechanism

Lineus ruber feeds mainly on small annelids and crustaceans but particles of any dead organic material will be taken, providing it is not too decomposed. The oligochaete *Clitellio arenarius* was particularly common in the habitat and at the time of collection (July–August) appeared to form the bulk of the food.

Living prey is detected by the eye-spots and a starved *Lineus* will respond to animals moving within 2 cm. of the head. Dead or injured animals emitting decomposition products or body fluids can be located at greater distances and here it is presumably chemoreceptors in the cephalic ciliated grooves which are stimulated. Detection of living prey is followed by immediate eversion of the proboscis[1] through the proboscis pore at the anterior tip of the body, and this occurs with such explosive force that as the proboscis strikes the prey it becomes coiled around it in a tight spiral grip. It does not penetrate the prey, since it lacks stylets or similar piercing organs, but the tightness of its grip may rupture the integument and cause loss of body fluids or gut contents. The grip is aided by sticky secretions from the proboscis epithelium and immediately it is secured the proboscis begins to retract and draws the prey, usually struggling violently, back towards the mouth. This lies ventrally 2–3 mm. behind the proboscis pore, and as the prey is drawn towards it the body anterior to the mouth is raised and extended until it can grasp the prey by curling downwards over it. This movement of the anterior tip of the body continues downwards and backwards and forces the prey into the mouth which gapes open to receive it. The proboscis then gradually relinquishes its grip and withdraws into the rhynchocoel as movements of the mouth, aided by contractions of the anterior body musculature, force the prey into the gut. Ingestion of small animals is completed in 15 to 20 seconds but with larger prey, such as annelids one-third to one-half the length of the feeding individual, it may take as long as 30 minutes, and in such cases the first part to be swallowed is partially disintegrated before ingestion is complete. Small animals usually die within seconds of entering the gut, but active errant polychaetes with armored jaws may survive long enough either to force their way to the exterior through the gut and body walls or propel themselves down the length of the gut to emerge unharmed at the anus. This is particularly liable to happen when the prey is ingested head first, but in the majority of cases the proboscis seizes an animal about its middle and consequently draws it back to the mouth bent upon itself in the shape of a U. It is then ingested in this form and is unable to escape from the gut before being killed.

During capture and ingestion *Lineus* extends to its fullest length and produces copious sticky mucoid secretions from the ventral surface. This enables a firm hold to be retained upon the substratum whilst dealing with the prey, and even if the latter is partially buried it can be drawn from its retreat and swallowed without causing the *Lineus* to shift position.

Inert masses of food, such as animal remains or the test foods used in this investigation, do not stimulate eversion of the proboscis but are seized directly by the mouth and swallowed piecemeal.

The structure of the gut

The gut consists of three histologically distinct regions, namely the mouth and buccal cavity, the foregut, and the intestine. It runs the length of the body from mouth to anus without coiling, is ciliated throughout and lacks both multicellular glands and musculature.

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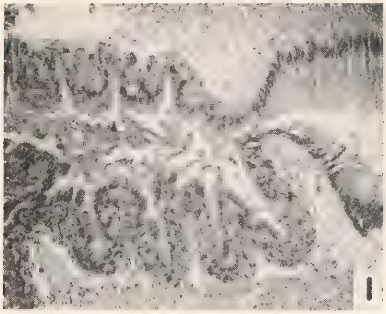
[1] Details of the proboscis and the mechanism of its eversion are given by Hyman (1951) and are not included here.

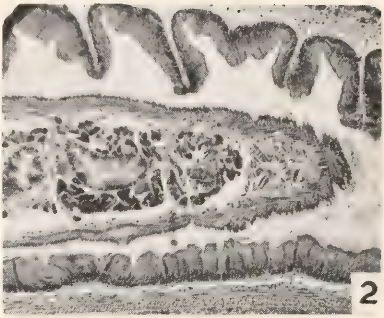
The mouth consists of a ventral subterminal invagination of the epidermis some 200 μ deep and opening directly into the buccal cavity. It is fringed with large cilia and externally has a lobed appearance due to folds in its walls which allow expansion during ingestion. The invaginated epidermis contains acidophil, P.A.S.- and Alcian blue-positive gland cells whose secretions probably facilitate passage of food, and the entire mouth region is surrounded by concentrations of similar sub-epidermal gland cells. The buccal cavity is lined by ciliated cuboidal cells 10–12 μ tall and these are backed by masses of acidophil and basophil gland cells, the majority of which stain with Alcian blue and appear to have the same function as those around the mouth. The walls of the cavity are much folded and ascend diagonally backwards to become continuous with those of the foregut beneath the proboscis sheath.

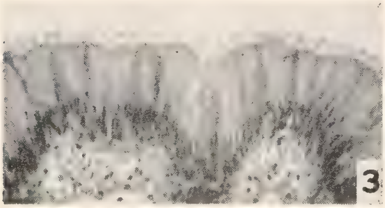
The foregut (Fig. 1) runs posteriorly for one-tenth the length of the animal and its walls are considerably thickened, especially ventrally where the wall may be up to 300 μ in depth. They are thrown into small simple folds and consist of a single layer of ciliated cuboidal cells, 10–12 μ tall, lining the lumen and lying upon acidophil syncytial tissue containing numerous gland cells, free nuclei and occasional large lacunae. In the anterior portion the gland cells consist of P.A.S.- and Alcian blue-positive acidophils and basophils in approximately equal amounts, together with a number of others which are intensely basophilic but give no reaction to Alcian blue. The proportion of the latter increases along the length of the foregut to the median portion where all the gland cells are of this type. The gut wall then gradually decreases in thickness and gland cell content as it nears the intestine and terminates in a constriction (Fig. 1) separating the latter from the foregut.

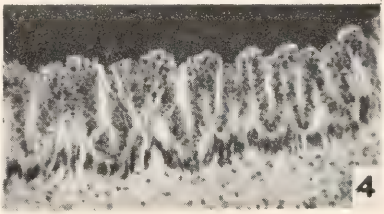
The intestine is the longest part of the gut and runs from its junction with the foregut in the anterior part of the body direct to the anus at the extreme posterior end. It bears paired and serially repeated lateral pouches or caeca throughout its length, apart from a short unpouched region immediately before the anus. The intestinal wall or gastrodermis (Fig. 3) is made up of two types of cells arranged in a single layer upon a thin basement membrane. The larger and more numerous of these are attenuated columnar cells, 50–55 μ tall and 8 μ wide, with granular basophilic cytoplasm containing various acidophil inclusions and basal vesicular nuclei. The free distal borders of the cells bear cilia which in unfed individuals are of uniform appearance and size, but in the presence of digesting food the cilia lose their uniformity and coalesce into pseudopodia-like processes which extend out into the lumen (Fig. 5). This peculiar modification of the cilia is correlated with entry of food material into the cells and is dealt with later.

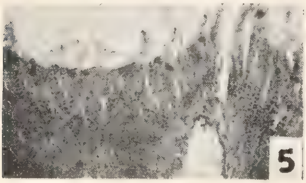
The second type of cell found in the gastrodermis is glandular and occurs between the bases of the columnar cells. These gland cells (Figs. 2 and 3) are 40–50 μ tall and 5–6 μ wide, unciliated and contain up to thirty acidophil proteinaceous spheres, each 0.5 μ in diameter, which are discharged into the intestinal lumen when food enters from the foregut. They are most numerous in the anterior part of the intestine, where there may be as many as one gland cell to every three columnar, but this ratio is graded down the length of the intestine to approximately one in twenty in the middle region and one in fifty or more beyond until the gland cells disappear, finally, in the short unpouched region before the anus.











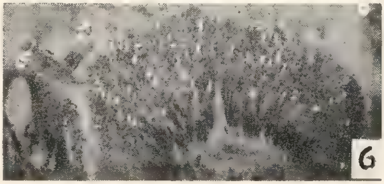


FIGURE 1. Longitudinal section of *Lineus* showing the posterior portion of the foregut (left) and the constriction which separates this from the intestine (right). Haematoxylin and eosin. Scale: 1 cm. = 50 μ.

FIGURE 2. Longitudinal section of the intestine in *Lineus* showing part of a newly ingested *Clitellio* lying intact and undamaged in the lumen. Acidophil gland cells are prominent in the gastrodermis in the lower portion of the figure. Haematoxylin and eosin. Scale: 1 cm. = 100 μ.

FIGURE 3. A portion of the gastrodermis in *Lineus* showing ciliated columnar cells interspersed with darker acidophil gland cells. Intestine empty. Haematoxylin and eosin. Scale: 1 cm. = 50 μ.

FIGURE 4. The gastrodermis in *Lineus* 30 minutes after a meal of frog erythrocytes. The intestinal lumen (top) contains a homogeneous mass of digested haemolyzed erythrocytes which stains heavily with Feulgen and almost obscures the ciliary processes. The columnar cells are loaded with engulfed spherical masses identical in nature with the contents of the lumen. Feulgen and light green. Scale: 1 cm. = 50 μ.

FIGURE 5. The gastrodermis in *Lineus* 30 minutes after a meal of raw starch grains. The cilia have coalesced into pseudopodia-like processes and a few starch grains already engulfed are ranged along the distal margins of the columnar cells. Lugol. Scale: 1 cm. = 50 μ.

FIGURE 6. The gastrodermis in *Lineus* 60 minutes after a meal of raw starch grains. The columnar cells are loaded with grains, many of which are as yet unchanged and still exhibit the characteristic black cross in polarized light. The lumen contains occasional free grains and on the left portions of two gregarine trophozoites with prominent nuclei. Section stained with haematoxylin and eosin and photographed by polarized light. Scale: 1 cm. = 40 μ.

The lateral pouches have the same structure as the rest of the intestine and are merely simple extensions to increase its area, not specialized digestive caeca.

The course of digestion

Ingested food passes rapidly through the buccal cavity into the foregut where it is held for a few seconds before its passage intact into the intestine (Fig. 2). Living food usually dies during the brief pause in the foregut and this is due, no doubt, to acid secretions from the numerous basophilic gland cells present here, for when particles of fish muscle stained with bromo-cresol purple or chlor-phenol red were fed, their pH value fell from 7.0 to 5.5 as they passed through the foregut, and sections of newly fed individuals showed the majority of the glands to be discharged.

There is no trituration or break-up of the food in the foregut but digestion begins immediately it enters the intestine. A series of individuals fixed at intervals after ingestion of the oligochaete *Clitellio* showed that as early as fifteen minutes after feeding the gland cells had discharged their spheres and digestion was well advanced. The *Clitellio* lay in the main median portion of the intestine with the epidermis deeply eroded and the entire body starting to fragment. The cilia of the columnar cells were still uniform in appearance and apparently creating currents in the gut contents to distribute the fragmenting food, for pieces of tissue were already passing into the lateral pouches. Digestion progressed rapidly with time and 30 minutes after feeding the intestine contained a heterogeneous mass of heavily eroded pieces of tissue, intact and fragmented setae, nephridia (which resisted digestion for longer than other tissues and stood out from these with surprising clarity) and diatoms, algal chains, etc. released from the oligochaete gut. The gastrodermal cilia were now beginning to lose their uniformity and coalesce into pseudopodia-like processes stretching into the lumen of the intestine, whilst semidigested material from the latter was appearing as acidophil spheres in the distal portions of the columnar cells bearing these structures. These spheres passed back deeper into the cells and their number increased rapidly with time. Sixty minutes after feeding, the material in the lumen was almost homogeneous, with setal fragments and diatom cases being the only recognizable elements in it, whilst the columnar cells of the gastrodermis were packed with spheres of food undergoing intracellular digestion. The spheres decreased in size and affinity for stains (especially the Millon reagent for protein) as they passed down the cells to disappear finally in the basal region, the cells presumably then passing the products of digestion to other tissues whilst taking up more semidigested material distally until the lumen was emptied. This occurred some six hours after feeding and the amount of intracellular material then rapidly decreased. After a further three hours the columnar cells contained only a few acidophil inclusions, their cilia had resumed their normal shape and size, and the gland cells were again full of enzymatic spheres. Indigestible residues were collected in the short unpouched region of the intestine near the anus, being swept there, probably, by the reconstituted cilia, and observations on living specimens showed that they were expelled eventually by a sudden contraction of the posterior body musculature.

A parallel series of feeding experiments, using fish muscle stained with indicators, showed that the initial drop in pH as the food passes through the foregut is maintained in the intestine during digestion. In some cases it fell even further, to pH 5.0, and when sufficient indicator-stained material entered the columnar cells to be visible in neutral saline squashes, the intracellular digestion was seen to be progressing in a similarly acid medium of pH 5.0–5.5.

It was not clear from the *Clitellio*-fed series how semidigested material enters the columnar cells, but the pseudopodia-like appearance of the coalesced cilia and spherical compact form of the material when within the cells suggested a form of phagocytosis. This possibility was investigated by feeding *Lineus* on frog erythrocytes and raw, optically active starch grains made palatable by mixing with frog plasma, to ascertain whether such discrete particles were in fact taken into the columnar cells. In the series fed on erythrocytes, however, haemolysis occurred as they entered the intestine, the break-up including the majority of the nuclei, and 30 minutes after feeding the lumen contained a semidigested mass which gave a strong reaction with Feulgen, due to released nuclear materials, and with the benzidine-peroxide reaction for haemoglobin. The cilia had coalesced into the usual processes and many of the cells contained spherical masses with the same staining properties as the material in the lumen (Fig. 4). These apparently phagocytosed masses passed back into the cells as more appeared distally, and gradually decreased in size and their reaction to Feulgen and benzidine-peroxide as intracellular digestion progressed. Digestion of the blood meal was completed in six hours and the intracellular spheres disappeared without leaving residues of haematin or other insoluble pigments from the degradation of the haemoglobin.

Final confirmation of the occurrence of phagocytosis came from the series fed on starch grains. Thirty minutes after feeding the cilia had formed filamentous processes extending into the lumen, and a few intact grains, staining blue with Lugol and still exhibiting the characteristic black cross in polarized light, had already been taken into the cells and were ranged along their distal margins (Fig. 5). The number of such grains increased rapidly and 60 minutes after feeding packed the columnar cells (Fig. 6). Only grains 5 μ or less in diameter were engulfed and larger ones remained in the lumen where they gradually lost their optical activity, fragmented and stained brown with Lugol. The fragments then passed into the cells and joined the previously engulfed grains which were undergoing intracellular digestion, losing their identity and disappearing towards the bases of the cells.

Parasites of the gut

Approximately 75% of the *Lineus* examined contained in the intestine an acephaline eugregarine identified as *Urospora nemertes* (Koelliker). Trophozoites (Fig. 6) 150–180 μ long and 15–20 μ wide, with basophil, P.A.S.-positive cytoplasm and prominent nuclei, occurred in all parts of the lumen, and the intracellular stages, strikingly prominent with P.A.S., were common in the columnar cells. The gregarine did not appear to harm *Lineus* in any way, apart from a few occasions when infected columnar cells reacted against developing intracellular stages and caused them to degenerate into masses of yellowish brown crystals. Such cells then burst, either *in situ* or after being shed into the lumen, and the crystals were eliminated with the faeces.

The food reserves

Fat forms the principal food reserve in *Lineus* and occurs as intracellular globules up to 5 μ in diameter in the parenchyma and, to a lesser extent, in the columnar cells of the intestine. There are no specific protein reserves and the only demonstrable carbohydrate reserve is in the form of tiny granules of glycogen scattered throughout the parenchyma, musculature, and columnar cells.

DISCUSSION

The main points of interest in the nutrition of *Lineus ruber* lie in the feeding mechanism and the digestive processes. In the case of the former a simple but effective method of capturing the food, supplemented by slight modification of the anterior portion of the alimentary canal into a thick-walled glandular foregut for its reception and killing, enables this rhynchocoelan to prey successfully upon animals far more active and elaborate than itself. In this respect *Lineus* resembles the turbellarian flatworms where similarly simple feeding mechanisms make available prey ranging from protozoa to molluscs and tunicates (Jennings, 1957; 1959a). In the Turbellaria it is the pharynx which forms the principal element of the feeding mechanism and this organ is thus analogous in function to the rhynchocoelan proboscis as seen in *Lineus*. The only disadvantage apparent in the type of feeding found in *Lineus* is the possibility of escape by the prey before the secretions of the foregut can take effect but this is overcome, no doubt, in those rhynchocoelans which possess a proboscis armed with stylets and poison glands by killing or paralyzing the prey at the moment of capture.

Digestion in *Lineus* follows a pattern observed in other Acoelomata (Jennings, 1957; 1959b) in that both extracellular and intracellular processes are concerned, but here the intestinal wall is ciliated and consequently the semidigested food would be expected to enter by absorption. In fact, however, it enters by a form of phagocytosis, as is proved beyond doubt by the appearance in the columnar cells of starch grains which retain their form and optical activity after entry and so must have passed into the cells as solid discrete particles. This method of taking material into the columnar cells involves temporary modifications in the form and behavior of the cilia during the digestion of each meal, and the protoplasmic pseudopodia-like processes formed from the coalescence of neighboring cilia are probably concerned in the engulfing of semidigested food, although this has not been observed histologically. The need for the intestine to be ciliated probably arises from its length and the absence of musculature, which together create a need for some method of distributing fragmenting food in the early stages of digestion and collecting residues near the anus at the end. Contractions of the body musculature appear to be insufficient for anything but the final expulsion of the collected residues and hence ciliary currents are used. The reason for the retention of phagocytic uptake of food under these conditions is unknown, for there is no apparent reason why extracellular digestion should not be carried to a point where the semidigested food is soluble enough for absorption, and this presents an interesting subject for further investigations.

SUMMARY

1. The rhynchocoelan *Lineus ruber* feeds on small annelids and crustaceans which are captured by the unarmed proboscis and swallowed whole.

2. The alimentary canal is differentiated into three regions: a buccal cavity, a foregut where the prey is killed by acid secretions, and an intestine where it is digested.

3. Digestion is the result of both extracellular and intracellular processes and occurs in an acid medium of pH 5.0–5.5. The enzymes responsible for the initial extracellular breakdown come from gland cells in the intestinal wall and digestion is completed within the columnar cells of the latter. Semidigested food enters these columnar cells by a phagocytic process and this involves temporary modifications in the form and function of their cilia.

4. The food reserves consist of fat deposits in the parenchyma and, to a lesser extent, in the columnar cells of the intestine.

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HERMAPHRODITISM IN THE SEA SCALLOP, PLACOPECTEN MAGELLANICUS (GMELIN)

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While hermaphroditism is of common occurrence among normally dioecious mollusks, a careful search of the literature failed to reveal any published record of this condition for the commercially important sea scallop, *Placopecten magellanicus.* Consequently, observations on the occurrence of the phenomenon in this species are of some interest and importance.

Our first hermaphroditic sea scallop was collected on September 17, 1959, while doing routine investigatory work relative to the scallop fishery aboard the chartered scalloper *Whaling City* in the western part of Georges Bank (68° 45′ W. Long., 43° 03′ N. Lat.). September is within the spawning season for the sea scallop on Georges Bank (Posgay and Norman, 1958). However, spawning had not yet started at this locality and all specimens, including the hermaphrodite, were ripe and full.

After close macroscopic examination the hermaphroditic gland was fixed for histological study in Newcomer's (1953) fluid and stained either by means of the Feulgen reaction or with Harris' hematoxylin and eosin. Some Feulgen-stained sections were counterstained with light green (yellowish). Pieces of tissue were cleared in chloroform, embedded in paraffin, and sectioned at 10 micra. For comparison normal male and female gonads were likewise treated except that in these Bouin's fluid was the fixative.

In the normal gonad the size and shape are similar in both sexes. The single gonad is tongue-shaped and occupies most of that portion of the body ventral to the foot. It extends dorsally to form a thin layer over the surface of a portion of the digestive gland. The genital organ is large and plump when ripe but after spawning it becomes much smaller, shriveled, and quite flaccid. The sexual products are easily seen through the tissue of the gonads, the ova giving the female gonad a bright coral red appearance at maturity, the sperm producing a whitish-cream coloration in the male gonad.

The hermaphrodite mentioned above has the male and female parts located in different regions of the same gonad. The proximal part forms the ovary while the testis lies distal to it. The boundary between the two regions is indefinite with quite irregular islets of one tissue occurring within the tissue of the other (Fig. 1a). This is even more pronounced histologically (Fig. 2a). The gonad is unspent and, relative to the degree of development, compares favorably with normal unspent gonads.

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The ovarian follicles are closely crowded and are filled with mature ova which take the shape of polyhedrons because of their tightly packed condition. The lumina of the follicles are completely filled. Each follicle is lined on the outside by a single cell layer of squamous epithelium. Inside the follicles the individual ova are separated by a non-granular intracellular substance sandwiched between and separating the cell membranes. This matrix is rather uniformly distributed and of little variation in thickness. Actually, it may not be intracellular in the strict sense but a component of the cell membranes. Mature ova average about 45–50 micra in diameter in fixed material. Their germinal vesicles are large and clear, and contain a fine network of chromatin and several conspicuous nucleoli (Fig. 2b). Connective tissue or follicular cells are rarely found between either male or female follicles in this individual.

The testicular follicles are tightly packed and are covered by the same kind of squamous cell layer as found covering the ovarian follicles. Inside the follicle and adjacent to the covering epithelium is a layer of spermatogonial cells. This layer of primordial germ cells is one to several cells thick (usually two or three, but sometimes up to ten or more). Progressing inward toward the lumen of the follicle are the primary and secondary spermatocytes, followed by spermatids, then mature sperm at or near the center. The tails of the spermatids are usually located in the most central part of the follicle. In portions of the testis where spermato-

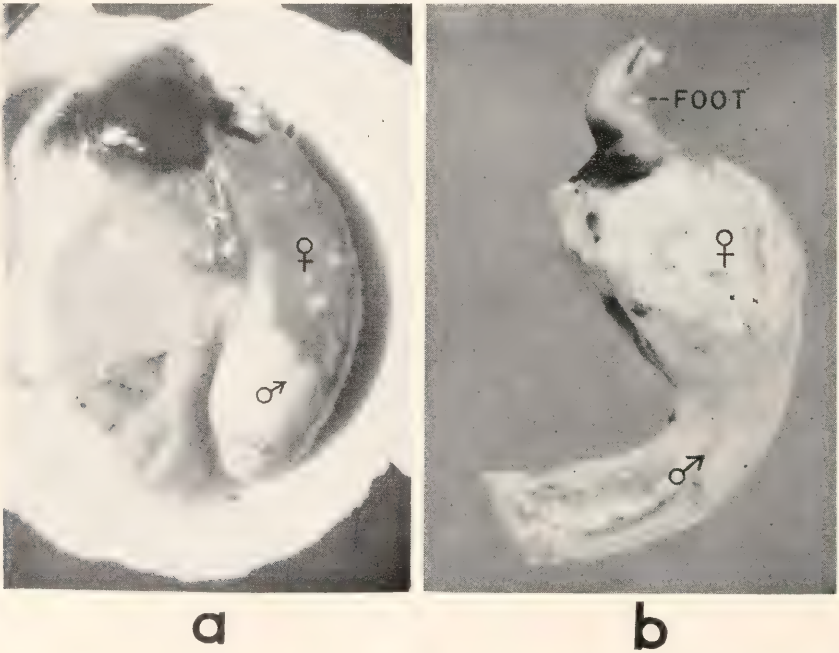

FIGURE 1a. Hermaphroditic *Placopecten magellanicus.* Mantle and gill tissue folded back to show the large bisexual gonad (× 2/3). 1b. Hermaphroditic gonad of *P. magellanicus.* Male portion has spawned, female (bulbous) part still unspawned (× 2). (Note: gonad fixed in Bouin's and preserved in alcohol before photo taken, this resulting in loss of color.)

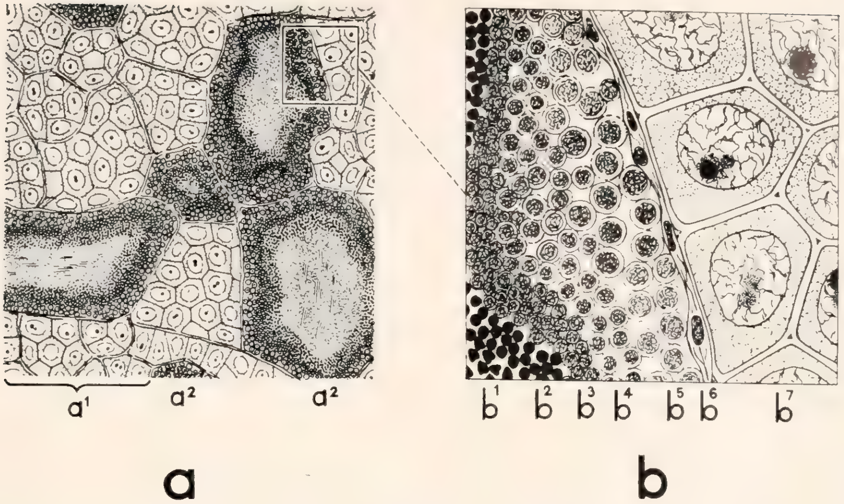

FIGURE 2a. Histological section through central portion of the hermaphroditic gland of specimen shown in Figure 1a (× 50). a^1, Portions of female follicles filled with ova. a^2, Portions of male follicles containing all stages of spermatogenesis. 2b. Enlarged portion of Figure 2a showing part of a male and a female follicle and their boundary (× 250). b^1, Mature sperm. b^2, Spermatids. b^3, Secondary spermatocytes. b^4, Primary spermatocytes. b^5, Spermatogonia. b^6, Epithelial lining. b^7, Portion of a mature ovum.

genesis is completed, the follicles are completely filled, from epithelial lining to epithelial lining, with mature sperm. However, even in such follicles, there are occasional cells at the epithelium which we take to be primordial germ cells but which may perhaps be follicle cells.

The number of spermatogonial divisions before synapsis can not be determined, but there is no evidence in this specimen, or in normal unisexual males, to suggest that there are not more than two (*e.g.*, see Coe and Turner, 1938, Fig. 17, p. 103). Since whole bunches of spermatogonia, ten or more cells deep, are sometimes seen to project out toward the lumen of the follicle, they probably go through a series of divisions before synapsis. In pulmonate gastropods, where the number of spermatogonial divisions can be accurately determined, there are normally six such divisions prior to spermatocyte formation (Burch, 1959).

Spermatogonia average about 5 micra in diameter in sectioned material, primary spermatocytes about 2.5 micra in diameter, and the width of each sperm head is about 1.7 micra.

The individual follicles in this hermaphroditic gland are either all male or all female; there are no ambisexual follicles. In the proximal end of the gland most follicles are female, and in the distal end most are male. But, in a wide central area both male and female follicles are widely and indiscriminately dispersed (Fig. 2a). The size and appearance of the cells, as far as can be ascertained, are identical to similar stages in normal individuals of this species. Atypical spermatogenesis as found by Coe and Turner (1938) was not observed.

A second hermaphrodite was found on November 21, 1959, in a sample from the eastern part of Georges Bank (66° 45′ W. Long., 41° 23′ N. Lat.). The arrangement of gonadal tissue in this specimen was similar to that of the first. However, the spermary was mostly spent while the ovary still remained large and plump (Fig. 1b) with no sign of having started to spawn. This differs from the observed spawning habit of the normally monoecious great scallop, *Pecten maximus*. In this species the products are released within a few hours of each other, with either the eggs or the sperm being shed first (Mason, 1958). Also, the arrangement of the genital tissues within the gonad of *P. maximus* is the reverse of that of the two hermaphroditic *P. magellanicus* (*e.g.*, the testis proximal and the ovary distal).

Of colleagues who have investigated various aspects of the scallop fishery, only Dickie[2] (personal communication) has advised of having observed this phenomenon. He remembered seeing hermaphroditic sea scallops on two separate occasions. Once during the summer of 1956 he was shown several preserved mature hermaphroditic gonads from Georges Bank by one of the scallop-vessel skippers. Another time (July 22, 1949) he found a 95-mm. predominantly female hermaphrodite from "Hour Ground" off Digby, Nova Scotia.

Hermaphroditism is the usual condition in most of the Pectinidae (Coe, 1945); *P. magellanicus* is an exception. Hermaphrodites in other unisexual genera of pelecypods are not uncommon and it seems likely that the occasional deviations in the developmental processes which produce these hermaphrodites are due to the failure of the sex-differentiating mechanism to function normally, as has been suggested by Coe and others. According to the classification of Coe (1942) these abnormalities would be termed *accidental functional ambisexuals*, in which the primary sex factors go astray and fail to activate or suppress either the male or female hereditary mechanism at some early stage of development. This results in various amounts of both kinds of tissue being produced.

Young's (1941) suggestion that hermaphroditism in normally bisexual species may be due to aberrant chromosomal behavior during gametogenesis has not yet been corroborated by cytological or experimental evidence. Similar suppositions were advanced to account for production of male and female cells in the ovotestis of the pulmonate gastropod *Lymnaea stagnalis appressa* (= *L. s. jugularis*) by Crabb (1927) and in *Physa gyrina* by Mahoney (1940). Perrot (1930), however, showed that this was not the case for *L. stagnalis* and Burch and Bush (1960) have shown the observations on *P. gyrina* to be in error.

In the commercially important bivalves in which sex has been extensively studied, and which are dioecious, occasional occurrence of hermaphrodites is normal. Thus, to list a few examples common to the North Atlantic coastline, Thorson (1936) remarked that *Mytilus edulis* has a considerable percentage of hermaphrodites. Loosanoff (1936) in his sexual studies of the quahog (*Mercenaria mercenaria*) found two cases of functional hermaphroditism among several hundred mature clams. Also, Turner[3] (personal communication) mentioned having seen a functional hermaphrodite in this species. He observed it to release the sperm first, and then the eggs. Coe and Turner (1938) found three cases of hermaphroditism on examining about a thousand soft-shelled clams (*Mya arenaria*). In the case of *P. magellanicus* about 3000 gonads were inspected after the first and before the second hermaphrodite was found. The low frequency of occurrence accounts for this condition seldom being observed in the sea scallop.

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[3] Harry J. Turner, Jr., Marine Biologist, Woods Hole Oceanographic Institution.

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ANTIGENS OF ARBACIA SPERM EXTRACTS[1]

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The initial steps in fertilization appear to involve interactions of the sperm and egg surfaces at the molecular level (see Tyler, 1948, 1949; Metz, 1957a, 1957b). Most of the present information concerning such interaction has been obtained from studies of egg and sperm extracts. Among sperm extracts those with action on eggs have commanded most interest. In the sea urchin and certain other forms, extracts prepared by a variety of methods precipitate the egg jelly layer, agglutinate eggs and neutralize the sperm agglutinating action of the fertilizin obtained from eggs. These effects of the extracts may result from the action of the sperm-surface receptor substance, antifertilizin, with which fertilizin combines in the sperm agglutination reaction (*e.g.* Tyler, 1948; Metz, 1957b). Whether or not these effects are to be identified with antifertilizin, absorption experiments have shown that such extracts contain some antigens in common with those of the sperm surface (Köhler and Metz, 1959a, 1959b, 1960). Further examination to reveal a more complete spectrum of antigens in such extracts seemed desirable. Accordingly, in the present investigation, *Arbacia* sperm extracts were examined for antigens by means of agar diffusion and immunoelectrophoretic techniques. The study revealed a maximum of four antigens in extracts prepared by freeze-thawing sperm.

Material and Methods

Arbacia punctulata from the vicinity of the Florida State University Marine Laboratory, Alligator Point, Florida, and from Woods Hole, Massachusetts, were used in the study. Semen was usually obtained from the animals by electrical stimulation (Harvey, 1956). The spermatozoa were separated from the seminal plasma by centrifugation (approximately 3000 × *g*; 20 minutes) at 4° C. The packed sperm were resuspended once in sea water and settled again by low speed centrifugation. The final supernatants following such washing regularly failed to give precipitation bands when diffused against anti-sperm serum. Standard sperm suspensions were prepared by diluting the packed sperm with three volumes of sea water.

Sperm extracts were prepared from such suspensions of washed sperm by a variety of methods. These included the established methods for preparing agents

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which act upon the egg jelly layer, namely, heating sperm to 100° C. (Frank, 1939) and freeze-thawing sperm (Tyler, 1939). The latter extracts are called "frozen-thawed extracts" below. Other methods are described with the individual experiments.

Antisera were prepared by injecting rabbits with sperm (25% washed sperm in sea water). The immunizing antigens were administered through intravenous, intraperitoneal and subscapular injections. In the last instance the antigen was injected as an emulsion in Freund's adjuvant (Difco). Several anti-*Arbacia* sera were examined. With the exceptions noted in the text the experiments reported here were performed with serum from the hyper-immune rabbit "#33." This rabbit received three injections of antigen in Freund's adjuvant (Difco) over a period of five months and was bled two weeks subsequent to the final injection. The immune serum obtained regularly agglutinated sperm to dilutions of 2^{-8} to 2^{-10}. No sera were pooled.

Agar diffusion and electrophoresis. Agar diffusion experiments using the technique of Ouchterlony (1948) were performed in 2% agar containing Merthiolate (0.01%) as a preservative. The reaction plates were incubated at room temperature for several weeks. Immunoelectrophoretic analysis (Wunderly, 1957) was performed using 2% agar blocks prepared in 0.05 ionic strength veronal buffer, pH 8.5 and containing 0.01% Merthiolate. Wells in the agar block were filled with antigen prepared as follows: after dialysis against 0.05 ionic strength veronal, the antigen was heated to 45° C. and mixed with an equal volume of melted (45° C.) 4% agar, also in veronal buffer. The mixture was then pipetted into the antigen wells of the agar slab and allowed to solidify. Agar slabs measuring approximately 20 × 6.05 cm. with antigen wells of 0.3–0.4 ml. capacity were used in these experiments. To achieve electrophoretic migration the preparations were subjected to a current of 25 ma for about six hours.

To improve the resolution of precipitin bands the agar blocks were fixed in 2% acetic acid, stained with Amidoschwarz (0.1% in acetate buffer, pH 4.0 solution) and destained in methanol-water-acetic acid (45:45:10).

RESULTS

Agar diffusion precipitin tests were performed on extracts prepared by freeze-thawing washed *Arbacia* sperm and subsequently centrifuging the extracts in a clinical centrifuge (approximately 3000 × *g*). When diffused against anti-whole sperm serum such extracts yielded a maximum of four precipitin bands. Proceeding from the antigen to the antibody well in the agar plate, these four bands are designated a, b, c, and d. As seen in Figure 1, some variation in band number was found in repeated tests (*e.g.* two bands in Figures 1a5, 1b2, 1b3; three bands in 1b4, 1c6; four bands in 1c1, 1d3). Antisera other than #33 gave one to two bands. These differences in tests with different "frozen-thawed extracts" using serum #33 are attributed to differences in antigen concentration. However, the possibility of qualitative differences has not been eliminated. Differences in the band number using other antisera may reflect differences in concentration of antibody as well as antigen in the tests. In view of the fact that the sperm were washed sufficiently before extraction to remove seminal plasma antigens, the precipitin bands in the extracts must have arisen from antigens extracted from the sperm cells.

In this connection it is of interest that undiluted seminal plasma forms three bands when diffused against anti-whole sperm serum (Fig. 1a4, 1b1). Two of these seminal plasma bands join but do not cross two of the "frozen-thawed extract" bands. It appears, then, that the seminal plasma shares at least two antigens with the extract. In fact these seminal plasma antigens may have diffused from the sperm. However, the one band that has been clearly demonstrated in the supernatant of aging sperm is band *a* of "frozen-thawed extract" (Fig. 1c3). It should be noted that the three bands just described do not constitute the complete antigenic spectrum of *Arbacia* seminal plasma. As seen in Figure 2d, immunoelectrophoresis resolved seven bands in this material. Three of these correspond in position to three precipitin bands in the sperm extract (Fig. 2c).

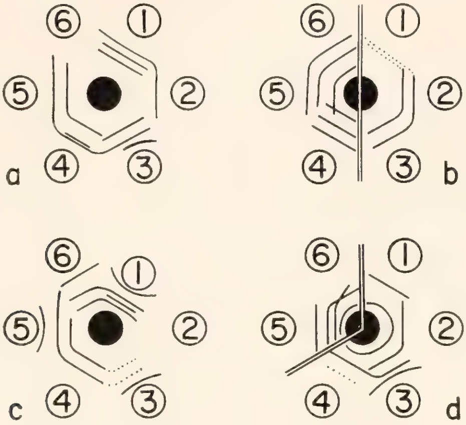

FIGURE 1. Ouchterlony agar diffusion tests. The center wells of all plates contain anti-*Arbacia* sperm serum No. 33. All surrounding wells were filled with extract and fluid of *Arbacia punctulata*. Each well received 0.5 ml. of the sample: (a) (1) supernatant over frozen-thawed sperm after centrifugation at 26,000 × *g* for 20 minutes; (2) residue of No. (1), resuspended in sea water and centrifuged at low speed; (3) supernatant over Mickle-disintegrated sperm; (4) seminal plasma; (5) "frozen-thawed extract" of sperm, low speed centrifuged; (6) body fluid; (b) (1) seminal plasma; (2) "frozen-thawed extract" of whole sperm, low speed centrifugation; (3) "frozen-thawed extract" of whole sperm, low speed centrifugation; (4) "frozen-thawed extract," low speed centrifugation; (5) supernatant over Mickle-disintegrated sperm, low speed centrifugation; (6) supernatant over Mickle-disintegrated sperm, low speed centrifugation; (c) (1) "frozen-thawed extract," low speed centrifugation; (2) basic protein, pH 0.9 extract; (3) supernatant over washed sperm after standing (aging), low speed centrifugation; (4) supernatant over citric acid-extracted sperm, low speed centrifugation; (5) supernatant over urea-treated sperm; (6) frozen-thawed extract, low speed centrifugation; (d) (1) isolated heads, "frozen-thawed extract," low speed centrifugation; (2) isolated tails, "frozen-thawed extract," low speed centrifugation; (3) whole sperm, "frozen-thawed extract," low speed centrifugation; (4) isolated heads, "frozen-thawed extract," low speed centrifugation; (5) acid extract (pH 3) of sperm, low speed centrifugation; (6) acid extract (pH 1.9) of sperm, low speed centrifugation.

It should be clear from these results that extracts prepared by freeze-thawing sperm are not solutions of a single macromolecule. On the assumption that each precipitin band represents a single antigen, the extracts can contain up to four different antigens. Extracts of sperm prepared by other procedures also contained antigens. As seen in Figure 1d5, extraction of *Arbacia* sperm at pH 3 yielded a preparation which produced four precipitin bands. Evidently two of these antigens are labile to or are insoluble in stronger acids for, as seen in Figure 1d6, an aliquot of the same sperm suspension extracted at pH 1.9 yielded but two precipitin bands. Extraction at even lower pH (pH 0.9) yielded preparations that failed to form precipitin bands. The extracts prepared by extracting *Arbacia* sperm at pH 3 failed to precipitate egg jellies.

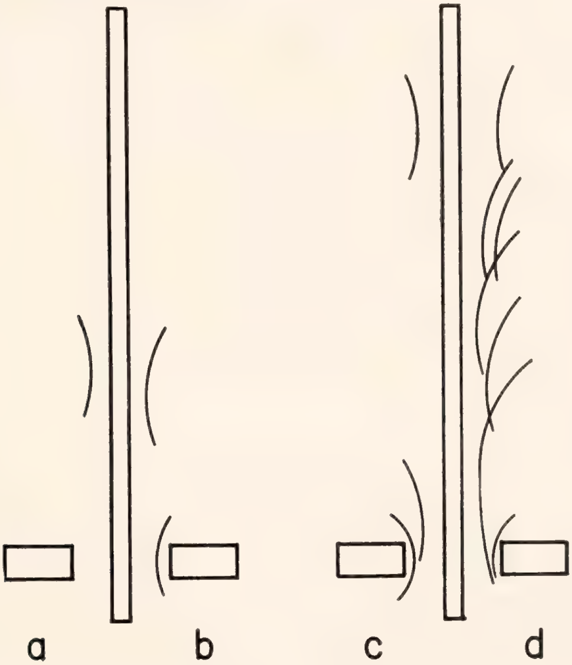

FIGURE 2. Immunoelectrophoresis in agar of various fluids from *Arbacia.* The central channel contains anti-*Arbacia* sperm serum No. 33, lateral channels contain preinjection serum No. 33. (a) Heated sperm extract; (b) "frozen-thawed extract," low speed centrifugation; (c) "frozen-thawed extract," low speed centrifugation; (d) seminal plasma.

Likewise, extracts prepared by treatment of sperm in the Mickle disintegrator (20 minutes, 20° C.) yielded three precipitin bands (Fig. 1b5 and 1b6). These have not been homologized with the antigens of "frozen-thawed extract." However, two (Fig. 1b4 and 1b5) of the bands correspond in position to two of the bands from "frozen-thawed extract." The third band crosses one of the bands of the "frozen-thawed extract" (probably the *d* band) and may represent an antigen not present in "frozen-thawed extract." Other extracting agents used were 1/18 *M* Na-citrate and 4 *M* urea. As seen in Figures 1c4 and 1c5, citrate extract produced two bands and urea extract three. The two citrate extract bands join two of the three urea extract bands. The third band of the urea extract is probably identical with the *a* band of "frozen-thawed extract."

As might be expected, extracts prepared by heating (100° C.) *Arbacia* sperm for one to five minutes do not have the full complement of soluble antigens. Such extracts at most yield a single band when diffused against anti-whole sperm serum. This antigen is evidently present in low concentration in extracts prepared by heating 25% sperm suspensions, for most such preparations fail to produce any precipitin bands in Ouchterlony or immunoelectrophoretic tests (*e.g.* Fig. 3a3). The band that does appear corresponds in position to one of the immunoelectrophoresis bands of "frozen-thawed extract" (see Figure 2 and below).

As seen above, extracts of whole sperm can contain at least four precipitating antigens. As in the case of the sperm agglutination antigens (Köhler and Metz, 1960), it seemed of interest to attempt to determine if these are present in both sperm heads and tails. Accordingly, sperm were broken into heads and tails by Mickle disintegration, these structures were separated by differential centrifugation and finally extracted by freeze-thawing (for details, see Köhler and Metz, 1960). When examined for precipitating antigens by agar diffusion, the frozen-thawed whole sperm extract produced four bands (Fig. 1d3). Head and tail extracts, adjusted to corresponding concentration, each produced two bands. One of these was common to both extracts and joined the *d* band of whole sperm extract. The second band in the head and tail extracts also joined bands produced by whole sperm extracts. However, these bands were not (Fig. 1d4) identical. The second band from the head extract joined the *b* band of whole sperm extract whereas the second band of tail extract joined the *c* band of the whole sperm extract (Fig. 1d2).

From the foregoing, it appears that both head and tail extracts lack band *a* of whole sperm extract. The absence of band *a* in the head and tail extracts is attributed to loss of this readily extracted antigen in the isolation process. Band *b* is present in extracts of isolated heads, but not tails; band *c* is present in tails but not heads and band *d* is present in both head and tail extracts.

Immunoelectrophoresis. In attempts to further resolve the antigenic composition of sperm extracts and seminal plasma these were subjected to immunoelectrophoresis in agar blocks (2% agar in 0.05 ionic strength veronal, pH 8.6). This method resolved three precipitin arcs (in three separate experiments) in frozen-thawed sperm extracts. As seen in Figure 2, b and c, one of these moved rapidly toward the anode, a second moved with intermediate speed and the third did not move appreciably. In comparative studies seminal plasma produced seven definite arcs (Fig. 2d). Three of these corresponded in position to the three arcs of the frozen-thawed sperm extract. Heat-extracted sperm (100° C., one minute) never produced more than a single arc (Fig. 2a). This corresponded in position to the fastest migrating antigen of unheated extract and seminal plasma.

Experiments with extracts centrifuged at high speed. In the studies presented above (Figs. 1 and 2), the extracts of frozen-thawed sperm were centrifuged at low speed (*e.g.* 3000 × *g*) only. Upon high speed centrifugation (*e.g.* 26,000 × *g*) of such extracts a pink, semi-gelatinous pellet is obtained and much, if not all of the egg jelly precipitating activity of the extracts is associated with this sedimentable material (Köhler and Metz, 1959b). Therefore, a comparative study of extracts before and after such high speed centrifugation was undertaken. This showed that three of the bands (b, c, d) remained in the supernatant of "frozen-thawed extract" after centrifugation at 26,000 × *g* (Fig. 3a1 and 3a4). The *a* band was absent from such preparations. The pink pellet obtained after the high speed centrifugation was homogenized in a small amount of sea water, and gave only the *a* band when diffused against anti-sperm serum (Fig. 3a2 and 3a6). This result suggests that the *a* band (e.g. in Fig. 1a3, 1c1, 1c3 and 1d3) represents antigenic material, that is readily sedimented by high speed centrifugation.

In comparative studies with extracts prepared by other means and centrifuged at high speed, frozen-thawed and acid extracts (pH 3.2) showed one band (probably the *c* band) in common (Fig. 3a5). In a second experiment (Fig. 3c and 3d) only two bands were resolved in the high speed supernatant of "frozen-thawed extract." One of these (probably the *b* band) is common to the high speed supernatants of seminal plasma (Fig. 3c3) and heat extract (Fig. 3c6) and the low speed sediment of the original "frozen-thawed extract." Histone prepared by pH 0.8 extraction of sperm and nucleoprotein prepared according to Mirsky and Pollister (1942) gave no precipitation bands with the antiserum after high speed centrifugation (Fig. 3d). This is not surprising since histone and nucleoprotein are generally found to be poor antigens (Cushing and Campbell, 1957).

The supernatant obtained after high speed centrifugation of "frozen-thawed extracts" was also compared with extracts obtained by aging sperm (48 hours, in sea water), extraction with 4 *M* urea and with 1/18 *M* citrate for 48 hours (Fig. 3b). One band, possibly the *c* band, is common to all of these extracts.

DISCUSSION

The results reported here show that extracts of frozen-thawed *Arbacia* sperm can yield four distinct precipitation bands when diffused against anti-whole sperm serum. These four bands are interpreted as four separate antigens. Detection of all of these antigens in agar diffusion precipitin tests appears to depend upon having high titer antisera and concentrated solutions of extract. Indeed these antigens may be only slightly soluble substances. Other extraction procedures (*e.g.* heating, acid, citric acid, and urea extraction) have not clearly revealed additional antigens. Likewise immunoelectrophoretic analysis revealed three antigens in frozen-thawed extracts. It appears, then, that only a few sea water-soluble antigens are obtained from *Arbacia* sperm in appreciable concentration. Possibly immunization of additional rabbits or other animals might yield an antiserum of unusual resolving power and reveal additional sperm antigens. However, the experience so far suggests that but few additional antigens would be discovered. The relatively small number of antigens in sperm extracts is in sharp contrast to *Arbacia* seminal plasma and egg extracts prepared by freeze-thawing. Parallel immunoelectrophoretic studies using anti-*Arbacia* egg sera and frozen-thawed extracts of *Arbacia* eggs readily resolved nine antigens. Likewise Perlmann (1953) found at least ten precipitin bands in agar diffusion tests using 0.15 *M* NaCl extracts of *Paracentrotus lividus* eggs. In preliminary tests no cross precipitin reactions were obtained between sperm extracts and anti-egg sera or the reverse.

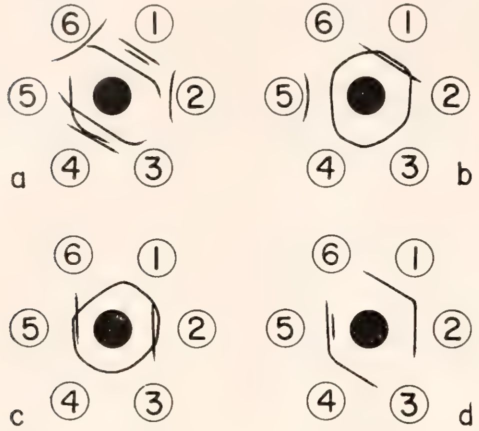

FIGURE 3. Ouchterlony agar diffusion tests. The center wells of all plates contain anti-*Arbacia* sperm serum No. 33. All surrounding wells were filled with extracts or fluids of *Arbacia punctulata.* Each well received 0.5 ml. of sample: (a) (1) "frozen-thawed extract" immediately subjected to centrifugation at 26,000 × *g;* (2) the pellet from No. 1 and No. 4, washed and resuspended; (3) heat extract, centrifuged at 26,000 × *g;* (4) "frozen-thawed extract" immediately subjected to centrifugation at 26,000 × *g;* (5) acid extract, centrifuged at 26,000 × *g;* (6) the pellet from No. 1 and No. 4, washed and resuspended. (b) (1) A very active egg jelly precipitating preparation obtained by resuspending the cake of frozen-thawed sperm followed by centrifugation at low speed; (2) 4 *M* urea extract of sperm, centrifuged at 26,000 × *g;* (3) 1/18 *M* Na-citrate extract of sperm, centrifuged at 26,000 × *g;* (4) supernatant over aged sperm centrifuged at 26,000 × *g;* (5) 4 *M* urea extract of sperm centrifuged at 26,000 × *g;* (6) 1/18 *M* Na-citrate extract of sperm, centrifuged at 26,000 × *g.* (c) (1) and (4) Supernatant over frozen-thawed sperm after high speed centrifugation; (2) and (5) the cake of frozen-thawed sperm was resuspended (same as Figure 3b1) in sea water for additional extraction (wells contain the supernatant after low speed centrifugation); (3) seminal plasma centrifuged at 26,000 × *g;* (6) heat extract, centrifuged at 26,000 × *g.* (d) (1) and (4) Supernatant over frozen-thawed sperm after high speed centrifugation; (2) and (5) the cake of frozen-thawed sperm was resuspended (same as Figure 3b1) in sea water for additional extraction (wells contain the supernatant after low speed centrifugation); (3) nucleoprotein centrifuged at 26,000 × *g;* (6) histone centrifuged at 26,000 × *g.*

Perlmann (1953), however, obtained one precipitin band when saline (0.14 *M*) extract of sperm was diffused against anti-egg serum.

The relationship, if any, of the four soluble sperm antigens to the sperm surface and to the egg jelly precipitating activity of the extracts has not been examined in detail. However, it is likely that the three antigens, *b*, *c*, and *d*, of "frozen-thawed extracts" are not related to the egg jelly precipitating activity because the latter activity is removed by high speed centrifugation whereas the *b*, *c*, *d* antigens are not sedimented by such centrifugation (Köhler and Metz, 1959b). With regard to the relationship of the soluble antigens to sperm surface antigens it should be recalled that absorption of anti-sperm serum with "frozen-thawed extracts" lowers the titer but does not completely neutralize the sperm agglutinating action of such antiserum (Köhler and Metz, 1960). This shows that the extracts contain some but not all of the sperm surface antigens. Agar diffusion experiments employing sera absorbed with whole sperm might reveal whether the soluble antigens are surface or subsurface material.

SUMMARY

1. Extracts were prepared from *Arbacia* sperm by several procedures. These were tested for antigenic composition by diffusion against anti-*Arbacia* sperm rabbit serum on Ouchterlony plates and by means of immunoelectrophoresis in agar gel.

2. Extracts prepared by freeze-thawing the sperm followed by low speed centrifugation produced a maximum of four precipitin bands. It is concluded that such extracts contain at least four soluble antigens.

3. Seminal plasma revealed seven arcs in an immunoelectrophoretic experiment, and a maximum of three bands on Ouchterlony plates. Two such bands join bands from "frozen-thawed extracts."

4. Extracts prepared by heating sperm at 100° C. yielded at the most one band. This antigen seemed to be common to several other extracts.

5. Nucleoprotein (Mirsky and Pollister) and histone failed to form precipitin bands.

6. One of the four bands in frozen-thawed extract is associated with material sedimented at 26,000 × *g*.

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SWIMMING SOUNDS AND THE SCHOOLING OF FISHES[1, 2, 3]

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This paper deals with the swimming sounds of some Bermuda fishes; it describes these sounds and relates them to the behavior of free fishes. E. V. Shishkova (1958a, 1958b) has recently published spectrograms of the sound produced by the movement of schools of scad (*Trachurus trachurus*, Carangidae) in the Black Sea. Such sounds are of interest in their possible significance to the behavior of fishes at sea, and as a reflection of fish behavior. The swimming sounds of fishes offer a possible means of control of fish school movements. The laughter which Hérubel derived (1912, p. 127) from the idea of controlling schools of herring is gradually fading. Sounds engendered by clupeids and the closely related engraulids are hitherto unreported, although a questionable account of herring sounds exists (Murray, 1831), and Maine fishermen describe sound stemming from large herring schools.

"Swimming sound" here conveys that collection of noises created in the water by movements of fish bodies, which characterize the movements of a given species. Such sound may stem from the rubbing together of skeletal parts, from the contraction of swimming muscles, or from water disturbed by various body movements. Shishkova refers to the swimming sound of *T. trachurus* as "hydrodynamic noise," which term she also applies to that noise caused by water flowing along a ship's hull; her data do not permit determining to what extent other events than water flowing over fish bodies contribute to the sound she describes. Since a similar criticism applies to my data, the more general term "swimming sound" seems preferable. Swimming sound is incidental to swimming and includes only those noises characteristically produced during swimming of a given species.

Listening equipment used in the investigation consisted of an AX-58-C Rochelle salt hydrophone and a Woods Hole Suitcase amplifier. Recordings were made during the summer of 1958 on a PT6-BN and a PT6BA2HZ Magnecorder tape recorder at 3¾, 7½ and 15 inches/sec., and analyzed on a Kay Vibralyzer vibration frequency analyzer. Recordings were played back into the water with an Ekotape tape recorder Model 205, a Craftsman C550 amplifier and a QBG transducer. Recordings at sea were made from the motor launch ABUDEFDUF of the Bermuda Biological Station, Mr. Brunell Spurling, Captain.

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[1] Contribution No. 1126 from the Woods Hole Oceanographic Institution.

[2] Contribution No. 271 from the Bermuda Biological Station.

[3] The work was performed at the Bermuda Biological Station and at Bowdoin College under Grant NSF-G4403 of the National Science Foundation, and through assistance of the Woods Hole Oceanographic Institution and of the Bowdoin College Faculty Research Fund established by the Class of 1928.

SWIMMING SOUNDS OF SOME BERMUDA FISHES

Anchoviella choerostoma (Goode)—Hog-mouth fry. This small engraulid occurred in large and small schools on Bermuda shores during the summer of 1958. The schools were most commonly seen in shallow bays along the north shore of St. George's and Hamilton Islands where they are said to be driven by the commonly observed activity of predators (chiefly carangids and lutianids) feeding on the schools (B. Spurling, personal communication). *Menidia* (Atherinidae) is probably similarly driven on Long Island shores (Butner and Brattstrom, 1960). E. L. Mark (1905) described some Bermuda fishes as gathering schools of smaller fishes to lure prey for the former.

Once established in a bay, a school may remain for several days and even for weeks, gradually reducing in size under peripheral predation and the depredations of fishermen obtaining bait for which the fry are highly valued. A large school darkens the water so that it may be seen over a considerable distance, and the experienced Bermuda boatman can readily distinguish between that discoloration caused by a fish school and that due to rock formation and weed patches.

Observations on the behavior of a large school at sea, estimated to contain several million individuals, of *A. choerostoma* were obtained mainly in Bailey's Bay on August 7, 1958. The launch was repeatedly brought over the school which moved in and out relative to shore during recording. Recordings were obtained when the sea surface was calm and the only extraneous noise stemmed from the system and from snapping shrimp.

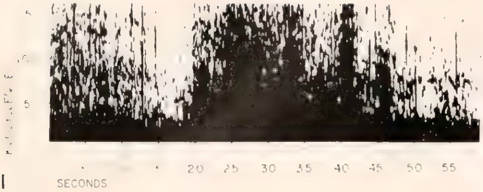
No predators were seen feeding during recording; activity around the borders of *A. choerostoma* schools on other occasions consisted of feeding by jacks (*Caranx* spp.) and pompano (*Trachinotus palometa*). Small *Caranx latus* (2 to 6 inches) were frequently netted with *A. choerostoma* and individuals were tolerated as members of small schools of the latter (up to 1000) maintained in the laboratory; the jacks fed on the host schools. *Jenkinsia* and *Sardinella* (Clupeidae) were also taken with *Anchoviella* on occasion.

For recording, the launch drifted over the Bailey's Bay school, engine off; although not obviously so, the behavior of the school was probably modified to some extent by the presence of the boat. During recording, the school was either lying at rest with individual fish turning slowly within a narrow circumference, or the

TABLE I

Characteristics of swimming sounds

Species	Size	Sound	Maximum frequency	Frequency of greatest intensity	Time duration
Anchoviella choerostoma	3 inches	Streaming	1.6 kc.	Below .5 kc.	Variable
		Veering of school	2 kc.	Below .8 kc.	.2-.6 sec.
Caranx latus	2–6 inches	Veering of individual	1.7 kc.	Below 1 kc.	.03 sec.
Caranx ruber	1 foot	Veering of individual	1.6 kc.	Below .7 kc.	.05 sec.
Trachinotus palometa	1 foot	Veering of individual	.7 kc.	Below .25 kc.	.06 sec.



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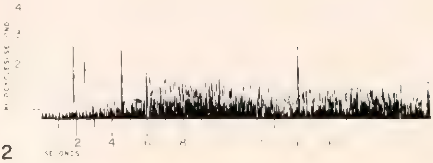

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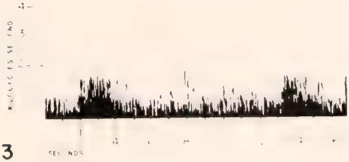

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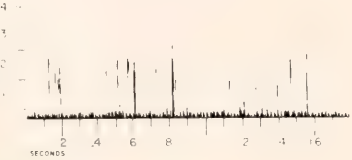

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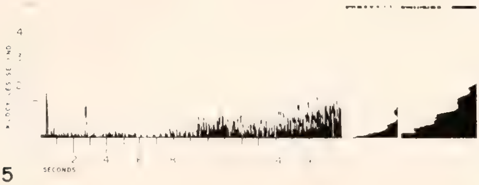

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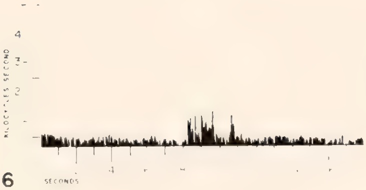

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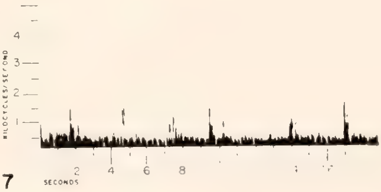

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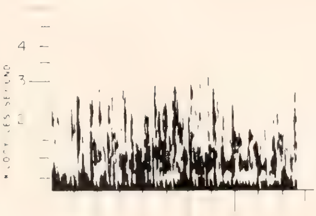

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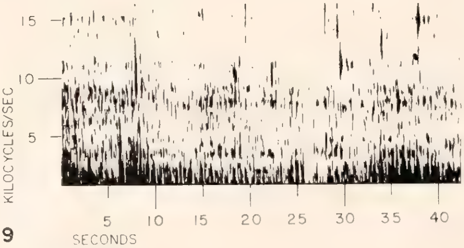

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Figure 1. Streaming, resting and veering of *Anchoviella choerostoma.*

Figure 2. Beginning of a streaming movement of *A. choerostoma.*

Figure 3. Two veerings during streaming of *A. choerostoma.*

Figure 4. Background and system noise during recording at sea in Bailey's Bay, August 7, 1958.

school had assumed a single direction and the fish were streaming around and by the hydrophone, or the school in the process of streaming suddenly veered in direction with a resultant sharp increase in sound intensity.

Sound could not be recorded from the school at rest; streaming and veering both produced considerable sound (Table I). Sounds similar to these are aptly described by Shishkova (1958a) for *T. trachurus* as being like a pouring or a splash and like a rise and fall in system noise. The greater the degree of veering, the greater the intensity of its noise. The school streamed slowly and rapidly past the hydrophone; sound intensity fluctuated with variations in speed of movement of the school.

Suitcase amplifier and Magnecorder settings varied from 0 + 4–10 and 20–3 db, respectively, to 0 + 14–7 and 30–3 db during recording; at all settings, some veerings of the school overloaded the system.

The entire school covered approximately half an acre; during the observation period, the school broke occasionally into two or three smaller groups. The school and its subdivisions tended to disperse somewhat and gather closely again, so that concentration of fish varied. At times, the school appeared layered, a thinner upper stratum moving in a different direction than the lower bulk of the school. All recordings were done over sandy bottom while the school lay in 2 to 4 fathoms of water; the hydrophone was lowered to mid-depth of the school. When streaming, the school moved past the hydrophone leaving a circular area clear of fish, extending about 6 inches from the casing. With a casing of different color, this interval might be expected to vary (Breder, 1951).

When the school was at rest, it could be stimulated to move by the slightest flexing and stiffening of the observer's knees aboard the 21-foot launch when visual clues were excluded. The casting of a shadow over the school by hand and body movements did not cause this behavior. If the school were at rest when pressure was applied to the deck of the boat by the knee-bend method, it would begin to stream; if it were streaming, it would veer and usually alter the speed of swimming to some extent.

The lower portion (up to 1.5 kc.) of a frequency analysis of sounds stemming from a typical sequence of movements of the *Anchoviella* school is shown in Figure 1. During the 6 seconds of recording illustrated, the school came nearly to rest, began moving again at 2 seconds, veered a few times between 2 and 5 seconds, then quieted again. Abbreviated vertical streaks in the background are due to snapping shrimp; some system noise is indicated below .25 kc.

A similar sequence of events is shown in Figure 2 over a broader frequency scale (5 kc.) and briefer time interval (1.7 seconds). Here the beginning of a streaming movement is shown, again with a few snapping shrimp spectra in the background. Figure 3 shows the spectra of two veerings of the school during a streaming movement. In each of these records, there has been sufficient attenua-

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FIGURE 5. Sections through spectrum of recording of *A. choerostoma* school while at rest and while streaming.

FIGURE 6. Veering of *Caranx latus* school.

FIGURE 7. Feeding and swimming sounds of *Caranx latus*.

FIGURE 8. Veering of *Caranx ruber*.

FIGURE 9. Veering of *Trachinotus palometa*.

tion to bring the schooling movements clearly into the foreground. In Figure 4, the ambient underwater sound of the area (mainly snapping shrimp) and system noise is shown after the *Anchoviella* school had moved inshore from the launch on one occasion.

Figure 5 shows sound intensity spectra (sections) during a recording made from the school at rest and after it had begun to stream. The first section was taken at .6 second of the time scale (school at rest), and the other at 1.3 seconds (school streaming). These sections provide a qualitative indication of the amount of sound stemming from a streaming movement at various frequencies at a point in time. Swimming sound of another *Anchoviella* school of considerably smaller dimensions could not be separated from background noise, due mainly to wave action during a moderate sea state with the equipment employed. In summary, *Anchoviella* swimming sound lies mainly below 2 kc. (Table I). Veering of the school introduces sharp intensity increases of between .2 and .6 second duration.

Attempts failed to record the swimming sound of *Anchoviella* in the laboratory from groups of 500 to 1000 individuals; the sound did not rise above extraneous noise. These experiments were performed in a cement tank, the fish being confined by a wood and plastic screen to an area 8 feet × 2 feet × 1 foot deep. The AX-58-C hydrophone was placed across the tank and fish driven around it. This paper presents evidence that the swimming activity of even small groups of *Anchoviella* is probably important in maintaining the coherence of the school; the swimming sound of a large school is one component of this activity.

A school of blue-fry (*Atherina harringtonensis,* Atherinidae) passing slowly under the launch on August 7 near Bay Island in Bailey's Bay did not respond to the knee-bend method successful in stimulating streaming and veering in *Anchoviella.* The blue-fry school was in the form of a compact ball of perhaps 1000 fish, each turning in its own particular pathway within the school as the latter passed beneath the boat. No swimming sound could be detected.

Caranx latus Agassiz and *C. ruber* (Bloch)—Yellow Jack and Skip Jack. These carangids, common in Bermuda, feed voraciously on *Anchoviella* as well as on other fishes. The *C. latus,* from 2 to 6 inches in length, were captured in small numbers with *Anchoviella* and separately off the dock of the Bermuda Biological Station. Observations are based mainly on aquarium experiments. Two large impounded schools of adult *Caranx ruber* were recorded on the west side of Coney Island on June 26 and August 6, 1958, through the kindness of a commercial fisherman (Mr. Spurling, Senior), and a third school was recorded during underwater listening in Castle Roads on July 11.

Since pharyngeal tooth stridulation may accompany the swimming sound of *Caranx* spp., this habit bears comment. Pharyngeal tooth stridulation of *Caranx hippos* has been described (Burkenroad, 1931; Fish, 1948; Moulton, 1958), and Fish (1948, 1954) describes the stridulating sounds of a number of other carangids. The pharyngeal tooth stridulation is produced more readily by young jacks hand-held gently under water (*C. latus, C. hippos*) than by adult jacks so treated (Moulton, 1958, p. 364); it was recorded at Bermuda from adult *C. ruber* held next to the hydroprone in an impounded school, and by *C. crysos* speared off Nonsuch Island on August 17.

Several attempts failed to elicit pharyngeal tooth stridulation from adult pompano (*Trachinotus palometa*, Carangidae) and *Calamus bajanado* (blue-bone porgy, Sparidae), both of which are equipped with formidable pharyngeal teeth. Adult grunts (*Haemulon sciurus*, Pomadasyidae) of some species, on the other hand, stridulate readily when similarly treated.

The present account deals mainly with the thumping sound produced by veering of *Caranx* spp., but pharyngeal tooth stridulation is also produced at times during this maneuver.

Several small groups (from 12 to 20) of young *C. latus* were confined in aquaria 1½ feet × 1¼ feet × 1 foot deep with the hydrophone. Undisturbed, these fish milled about the aquarium equidistant from each other, each on its own pathway. When a hand was flicked at the aquarium or a stick poked into the water, the small group of jacks became at once a tightly coherent school, streaming about the aquarium and veering on repetitive stimuli as had the *Anchoviella* at sea. Streaming did not introduce sound detectable above background; veering did. This movement even of individual fish resulted in distinct thumps similar to those described by Fish (1954) from shocked *C. crysos* (7.75 mm.?). When stemming from a group of *C. latus,* the thumps occurred in volleys (Fig. 6, Table I).

During feeding on bits of meat, the *C. latus* dispersed through the aquarium. The thumps then became scattered as individuals darted for food, and were accompanied by occasional sounds of the stridulation of teeth (Fig. 7).

During the Coney Island recordings, the hydrophone was hung in the midst of schools of *C. ruber* before and during raising of the net. Veering of the fish about the hydrophone caused volleys of thump-like sounds similar to those of *C. latus*, but somewhat deeper in predominant tone (Fig. 8, Table I). Swimming sound engendered by streaming movements was also detected in these recordings and that obtained at Castle Roads.

Trachinotus palometa Regan—Gaff-topsail Pompano. The swimming sound of two pompano approximately 1 foot long was recorded in the cement tank. The animals were not fed during recording. Only thumps coincident with veering occasioned by sudden hand movements in the water were recorded. Sound spectrograms of two flurries of activity are illustrated (Fig. 9, Table I).

Diplodus argenteus (C. and V.)—Bream. Attempts failed to record a swimming sound from 6 of these fish (Sparidae) approximately 8 inches long in a Station aquarium. The only sound detected was made by the fish bumping against the sides and bottom of the tank as a probe was moved gently about the aquarium. These sounds are not clear and sharp like carangid thumps; they are variable and random and are not clearly correlated with describable behavioral phenomena.

Various spear fishermen at Bermuda during 1958, including reliable observers from the Station, reported hearing sounds underwater produced during evasive swimming of grouper (Serranidae) and parrotfish (Scaridae), "any large fish," and hogfish (*Lachnolaimus maximus*, Labridae), as well as grunts of the hamlet or Nassau grouper (*Epinephalus striatus*) and volleys of squirrelfish (*Holocentrus ascensionis*) sound (Moulton, 1958).

The Playback of Underwater Sound to Fish Schools

The playback of underwater sound has been useful in modifying fish behavior (Moulton, 1956a, 1956b; Tavolga, 1956, 1958). The habit of *Caranx* of feeding on *Anchoviella* led to playing back to the *Anchoviella* school recorded on August 7 recordings of the stridulation sound of *C. latus* and of the swimming sound which accompanies its feeding. The transducer was lowered to mid-school level. The *Anchoviella* cleared the area beneath the launch more quickly during this playback than during recording. Further, during sound playback, the school showed a greater tendency to divide than during recording.

A collateral observation is of interest: When playback began of the stridulation sound at sea, an adult barracuda (*Sphyraena barracuda*, Sphyraenidae) came abruptly to a spot about 8 feet from the suspended transducer and lay quietly facing it for about three minutes. This behavior was unique to all observers, including Mr. Spurling, an experienced Bermuda fisherman and boat captain. (Mr. Spurling's comment: "I've never seen a barracuda act like that before.")

Stridulating sounds and thumps of young *C. latus* played back to about 500 *Anchoviella* in the Station cement tank were accompanied by quickened swimming and milling which gradually subsided. Approximately one-fourth of the fry moved to the opposite end of the tank over and under a horizontally suspended hydrophone which they did not pass spontaneously before sound transmission, and during hand movements in the tank.

Playback to the impounded school of *C. ruber* recorded at Coney Island on August 6, as it milled within the net, of a series of pharyngeal tooth stridulations of *C. latus* recorded in Station aquaria, initially was accompanied by an immediate movement of the school to the far end of the net. After some minutes of playback, the school failed to respond to these sounds and to feeding sounds. These sounds were listened to by surface divers and sounded like the amplified sounds heard during recording.

After the swimming sound of the large school of *Anchoviella* had been recorded at sea on August 7, the recording was played back into an aquarium containing young *C. latus*. The latter showed quickened swimming movements of a non-directional type. It has already been mentioned that small *C. latus* may join *Anchoviella* schools, feeding on the members of the school; they may be attracted to the school by its swimming sound.

Pharyngeal tooth rasps played back to the source species (*C. latus*) in a Station aquarium on August 1 appeared to initiate feeding reaction. The fish became exceedingly active, swimming about furiously, and facing the transducer to nibble at its rubber surface. After several minutes the activity subsided and the fish swam about as usual.

Swimming Movements and Schooling Behavior of Caranx and Anchoviella

The normal behavior of schooling fishes. *Caranx*, *Trachinotus*, and *Anchoviella* are truly schooling fishes, and whether at sea or in aquaria, groups of these fishes form cohesive schools. Their behavior bears examination as it relates to factors maintaining the school at sea.

When adult pompano attacked *Anchoviella* schools at sea, the former were in compact school formation, and usually swam directly through the school of fry; feeding of *Caranx crysos* at the periphery of a school was characterized by irregular darting movements and swift swimming of individual jacks.

At rest in aquaria and at sea, the *Anchoviella* school is clearly outlined, although individuals take independent pathways within the school in daytime as at night. When the school is streaming, and when a schooling fish (*Menidia menidia*) of the Woods Hole area is subjected to a tidal current in a live car, these fishes line up parallel to each other and veer together upon appropriate stimulus.

The importance of water currents in mediating behavior of fishes is further underlined by the behavior of small amberjack (2-inch *Seriola* sp., Carangidae) lurking under sargassum weed clumps southeast of Bermuda on August 18. The fish abandoned each clump as the 65-foot *Panulirus* drifted down upon it, and swam directly to another clump, often some yards away but always along the same line of weed and thus within the same convergent zone (Woodcock, 1944, 1950). Due to a combination of factors, significantly wind and Coriolis force, sargassum weed in the open sea tends to arrange itself in parallel rows, the rows being spaced at quite regular intervals under a given set of conditions, and parallel to wind direction (Woodcock, 1944, 1950). Water currents, probably even micro-currents, are of great importance in the open sea in determining the distribution of fishes (see Hasler, 1956); persistent sensory orientation to a particular current or moving body of water may be of considerable importance in maintaining not only the position but also the cohesiveness of fish schools, particularly through hours of darkness (Moulton, 1957a).

Members of a single school vary in their behavior. Schools of 500 to 1000 *Anchoviella* in the cement tank tended to divide at rest into two or three smaller groups; one group was usually markedly larger than the other(s). These groups, treated alike from capture, differed in sensitivity to stimuli; some were easily frightened, others held their position in the face of a variety of stimuli (*e.g.*, a prodding stick or hand).

In a study of variation in reactions to stimuli, a plastic screen confined a single school of about 1000 *Anchoviella* to an 8-foot section of the cement tank during attempts to record swimming sound. The hydrophone was placed across the tank, 3½ feet from the end containing the fish. During transmission of jack stridulation recordings in the area containing the fish, approximately one-fourth of the school moved past the hydrophone. Pushing down on a small board floating over the school (in simulation of the knee-bend method) caused approximately one-half the remaining fish to pass the hydrophone. Then a hand was waved vigorously in the water with the balance of the school; a recalcitrant two dozen fish still remained in the smaller portion of the tank, gathered in a cohesive school.

Young *Caranx latus* tend to school with *Anchoviella;* in an aquarium, a school of 12 of the former formed a tight formation immediately above the back of a 10-inch squirrelfish (*Holocentrus ascensionis*, Holocentridae). They turned with the squirrelfish as it moved slowly about the tank; the latter made no move toward the jacks. Another group of 20 *C. latus* was confined for several hours in an aquarium with an adult spiny lobster (*Panulirus argus*). So long as they were contained together, night and day, the *C. latus* remained at the top of the tank in a tight school. The spiny lobster was removed one night under artificial light, and at once the 20 jacks dispersed over the bottom of the aquarium and assumed a striking striped pattern completely unlike their usual drab coloration.

Experiments on blinded fish. In order to examine factors other than vision mediating the schooling of fish, adult *Anchoviella* were blinded by bilateral eye removal under anaesthesia (MS222 1:3000, Sandoz Pharmaceuticals) and allowed to recover, or became blinded through eye loss in a syndrome developing within hours of capture in a school held in the cement tank. On successive trials, a blind individual was placed in an aquarium with 12 normal individuals. When the latter were at rest, individuals milling slowly about, the blinded individuals swam through the group and about the aquarium generally until reaching the side of the tank, then took up new headings; they did not orient to the normal fish.

If the normal fish were startled by a hand movement next to the aquarium, they streamed and veered as did large schools at sea; at such times, the blinded individual immediately joined the normal fish in school formation and behaved as did the latter. Similarly, in the cement tank, blinded *Anchoviella* moved with the larger group of normal fish during streaming movements. When the normal fish came to rest again, blinded individuals returned to random movements, while even at rest the normal fish maintained approximately equal intervals between individuals. Isolated blinded fish did not respond to hand movements adjacent to the aquarium.

Unilaterally blinded *Anchoviella* maintained position with the normal fish when the latter were within the field of vision. This latter observation is in agreement with observations obtained on *Menidia* behavior at Woods Hole in 1957, where the experiments were performed in a live car suspended from a raft in Great Harbor; bilaterally blinded fish swam directly through groups of normal fish at rest and oriented independently of normal fish in currents flowing through the live car, while unilaterally blinded specimens maintained position with schools located at least in part on the side of the unoperated eye (Moulton, 1957a).

While movements of the individual members of the fish school are important in maintaining the remarkable integrity of the school, the members of the school are probably sensitive to pressure waves created by the school and probably orient to these waves; the movements of the school as a whole are probably important in maintaining its own integration.

Anchoviella did not disperse through the cement tank after dark as *Jenkinsia* (Breder, 1951) and chub mackerel—*Scomber colias*, Scombridae (Shlaifer, 1942)—do in aquaria. Schools of 500 to 1000 were in tight formation in the center of the cement tank in resting behavior during several examination periods on dark nights when an electric light was turned on, and behavior did not change notably with the light; the basement-located tank was too dark on moonless nights for me to see the fish in it.

C. latus in small numbers confined alone in darkness for several hours did not noticeably change their distribution when a light was turned on. Neither *C. latus* nor *Anchoviella* seems to depend so markedly on light for schooling formation as apparently do *Jenkinsia* and *Scomber*.

A Ghana Fishery Depending on Swimming Sound

A. P. Brown, in an anthropological account of the fishing industry of the Labadi District of the Gold Coast (Irvine, 1947, p. 25), mentioned the three-pronged paddles found in the sea-going canoes of the Coast, without indication as to their use. Inquiry was made subsequent to a United Nations news release suggesting an application to the fisheries.

The paddle, for possession of one of which I am indebted to Mr. D. A. Hammond of the Fisheries Department, Accra, is in typical form 4 feet 9 inches in length with a handle $1\frac{3}{4}$ inches in diameter. The foot-long flattened blade terminates in three broad, blunt teeth, comprising $4\frac{1}{2}$ inches of the blade length, each approximately 2 inches in width at the base, the center one tapering slightly more than the other two. Mr. Hammond describes the use of this paddle as follows:

"The use of the paddle as (a) hearing aid in fishing is well-known to our fishermen from Ada to Takoradi. It is particularly used by the herring fishermen. 'Herring' here refers to *Sardinella aurita* and *S. cameroonensis*. It is also used, however, to detect shoals of shad (*Ethmalosa dorsalis*), long-finned herring (*Ilisha melanota*) and cassava fish (*Cynoscion senegalla*). (Identifications from Irvine, 1947).

"Where there are no surface fish visible, the method is to place the broad part of the paddle in the water over the stern of the canoe, place the ear to the top of the handle, and rotate the paddle very slowly. Only skillful and experienced fishermen are appointed to do it. The face of the paddle acts as a sounding board and receives the vibrations which it transmits to the hearer. By rotating the paddle, the fisherman is able to get the direction of movement of the fish and from the intensity of the sound, he can judge how (distant) the fish (are). Those fish which move in large shoals—for example, herring or shad, as well as the large rocks in the sea, make distinct sounds. From the sound, the fisherman can tell whether it is a fish or rock. The sound of the herring is characteristic and never mistaken. This method is particularly successful in waters of 7 to 10 fathoms and between 11 P.M. and 3 A.M. Those paddles which are particularly good are treasured. Some of them are very old." A variety of the Ghana-type paddle is said to be used for a similar purpose in Liberia (Mr. William Watkins, personal communication).

Underwater listening occurs in various parts of the world as an important aspect of commercial fisheries (Cousteau, 1953; Kesteven, 1949; Marshall, 1954; Parry, 1954; Westenberg, 1953). It is likely that in the fishery described by Mr. Hammond, the swimming sound of the clupeids (sciaenids may produce other sounds) is conveyed by the three-pronged paddle to the fisherman's ear.

DISCUSSION

The schooling behavior of teleosts probably depends primarily on vision in the daytime (Shlaifer, 1942; Breder, 1951). Yet schools of *Anchoviella, Clupea harengus* and other schooling fishes maintain their schools at sea through hours of darkness; herring fishermen depend on this fact in "lighting up" herring schools at night (Moulton and Backus, 1955). While it may be that light stemming from luminescent organisms and that of moon and stars on clear nights may afford sufficient stimulus to abet schooling at night, some conditions of darkness at sea undoubtedly preclude vision and require other stimuli as predominant in providing for maintenance of the fish school at night. Certainly other factors than vision enable blinded fish (*Anchoviella, Menidia*) to move with their respective schools.

The possible role of water currents in maintaining fish schools at sea has been briefly discussed.

Significant factors in low frequency sensitivity of teleosts are the lateral line and isolated cutaneous receptors (von Frisch, 1938; Griffin, 1950; Lowenstein, 1957). Tracy (1920a, 1920b) and others have suggested that the connection between air bladder and inner ear of clupeids (engraulids possess a similar connection—Berg, 1947) may be more important as a pressure control device related to gas physiology of the air bladder than to hearing facility. While it is not unlikely that the paired passages may serve in both capacities, the remarkable sensitivity of *Anchoviella*, as well as of some clupeids (Moulton and Backus, 1955; Moulton, 1956a), to pressure waves in the water is suggestive of special adaptations to reception of pressure changes.

Anchoviella are sensitive to very slight pressure waves in the water; the swimming sounds described fall within the frequency range of sensitivity of all fishes studied in this connection. It is now simply conjectural, although some supporting evidence has been presented, to suggest that the swimming sounds of various carangids and of *Anchoviella* furnish a mechanism in addition to water currents and visual sensitivity by which schools of fish at sea are maintained under a variety of circumstances. Either these sounds or the body movements from which they stem must be a primary factor.

The swimming sounds described are to a degree species-specific. Shlaifer (1942) observed that chub mackerel, pairs of which will school together, will not school with abnormally moving fish of the same species. Normal *Menidia* and *Anchoviella* do not arrange themselves in schooling formation with blinded individuals; the latter will school with the former. It is suggested that the fish school at sea is maintained by a number of factors, given the proper amounts of food and minimum predation: sensitivity to particular water currents and masses, vision, and the behavior of the school as a whole which acts an an acoustical core and as a wave pressure source to which individuals of the schooling species orient. It is suggested that the swimming sound is not simply a mechanical or accidental sound, but that it possesses biological significance. It is demonstrated that source species and other species may react to amplified swimming sound.

Another factor influencing the maintenance of the fish school is reflected in differential sensitivity of *Anchoviella* to increasingly dispersive stimuli. After a series of such stimuli, a core of recalcitrant individuals remains in the test area in an experimental tank, and may furnish the nucleus around which more sensitive members of the school fluctuate at sea; streaming and veering may orient around a group of relatively insensitive individuals forming an "anchor" to the school.

It is clear that the swimming sound of different species of schooling fishes (*Anchoviella, Caranx, Trachinotus*) varies in its characteristics with species as well as with size of the fishes concerned. Large schools of fishes as small as *Anchoviella* (about 3 inches) and as large as *Caranx ruber* (about 1 foot) introduce considerable sound into the water. This may in future be utilized in experiments on schooling fishes in waters less clear than those studied, to test influence of various stimuli on schooling fishes and to explore sensitivity to these stimuli. Although Shishkova (1958a) abandoned the hope of stemming acoustically the flow of anchovies from the Russian Sea of Azov into the Black Sea, and of masking ship noise with swimming sound, other possible uses of swimming sound exist; a likelihood is present that fishermen can control acoustically to some extent the movements of clupeid and engraulid schools.

The characteristics of these sounds are more like those of sounds produced under circumstances related to defensive behavior of fishes than of sounds produced under other circumstances (Moulton, 1957b). The sound attendant on veering may have some protective value. If this were true, large schools of herring and anchovies would be more resistant to predation by other fishes than would small schools. The joining of *Anchoviella* schools by young *C. latus* may be primarily protective for the latter; their feeding on the host school would simply indicate poor "guestmanship."

The swimming sounds described, as well as sound stemming from the movement of any large fish in the sea, are probably due to a number of factors; hydrodynamic noise, to which Shishkova relates all of the sound, skeletal movements, and contraction of axial musculature during strong swimming movements against a gas-filled, resonating air bladder. None of the sounds described are continuous even during steady streaming movements, indicating that they stem from the bodies of individual fishes during muscle contraction, rather than from the hydrodynamic effects of continuous water flow around fish bodies.

All of the swimming sounds studied lie below 2 kc., and thus are of lower frequencies than many sounds created by fishes by organs apparently specialized for sound production (Moulton, 1958). These results differ from those of Shishkova (1958b) who found components of *T. trachurus* swimming sound at 16 kc. (It seems likely that her fish were chewing or stridulating during recording.) The frequency span of greatest intensity of swimming sound, as indicated by darkening of vibragrams, is lower for large fish than for small (Table I).

After submission of the manuscript of this paper for publication, the author's attention was called to a recent extensive study of social groupings in fishes by Breder (1959). Of special pertinence to the present work is Breder's caution that the responses of members of a fish school to light and darkness will vary with individuals, and that light exposure which fish have received earlier will be of great importance.

The author is grateful to Dr. Richard H. Backus for constructive criticism of the manuscript of this paper.

SUMMARY

1. The swimming sounds of four schooling species of Bermuda fishes are described. The sounds stem from streaming and veering of schools at sea.

2. Responses of schooling species to playback of sounds stemming from schools are described. Exploitation of these responses in experimental and commercial fishing is suggested.

3. On the basis of observation at sea and of laboratory experiments, mechanisms useful in maintaining formation of schools at sea are discussed. These include vision, water currents and pressure waves initiated by the movements of fish bodies and the sounds they engender.

4. An African acoustical fishery, probably relying on the swimming sounds of fishes, is described.

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BINUCLEATE AND TRINUCLEATE OOCYTES IN POST-OVULATION OVARIES OF RANA PIPIENS[1]

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The senior author had long been interested in the means by which diploidy can be produced in parthenogenetically developed frogs and larvae (Parmenter, 1920, 1925, 1933, 1940, 1952). He demonstrated (1952) the existence of the diploid chromosome number in three mature virgin eggs and considered this to be one source of diploidy with a known history of no delay in cleavage. He had found one instance (1940) of a diploid many-celled embryo in which, by direct observation, he knew that both polar bodies were given off and that it developed with no delayed cleavage. The discovery of a small number of binucleate ovarian eggs suggested another important possible source of such diploid parthenogenetic individuals.

In the course of undergraduate research in which nuclei were dissected from ovarian oocytes of *Rana pipiens,* several eggs were found which possessed two completely separate nuclei, and it was established that the binucleate condition was not an artifact of manipulation. The eggs used were in those stages where the nuclei had reached their fullest growth (stages 4, 5, and 6 of Duryee, 1950). Subsequently, a method was devised for scanning in ovarian tissue a large number of transparent oocytes in such early stages (stages 1 to 3 of Duryee, 1950) that the yolk would not interfere with direct observation of the cells *in situ.* It was hoped eventually that the chromosome content could be determined.

MATERIAL AND METHODS

Technique. The sac-like ovaries of female frogs in the post-ovulated condition were opened along their edges in Ringer solution by means of fine-pointed jeweler's forceps. The two halves, or sometimes individual pieces only, were floated onto a slide. They were scanned under low power (16 mm. objective) in the living condition, or were fixed in Bouin's fluid (which served as a stain), dehydrated, and mounted in damar. In all cases the slides were systematically surveyed and all eggs counted in which there were visible nuclei. Some cells were examined and measured first unfixed and then fixed.

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[2] All of the data herewith presented were collected and organized by Dr. Parmenter, assisted by Mr. Derezin. Unfortunately Dr. Parmenter died before the writing of the paper, which subsequently was undertaken by his wife.

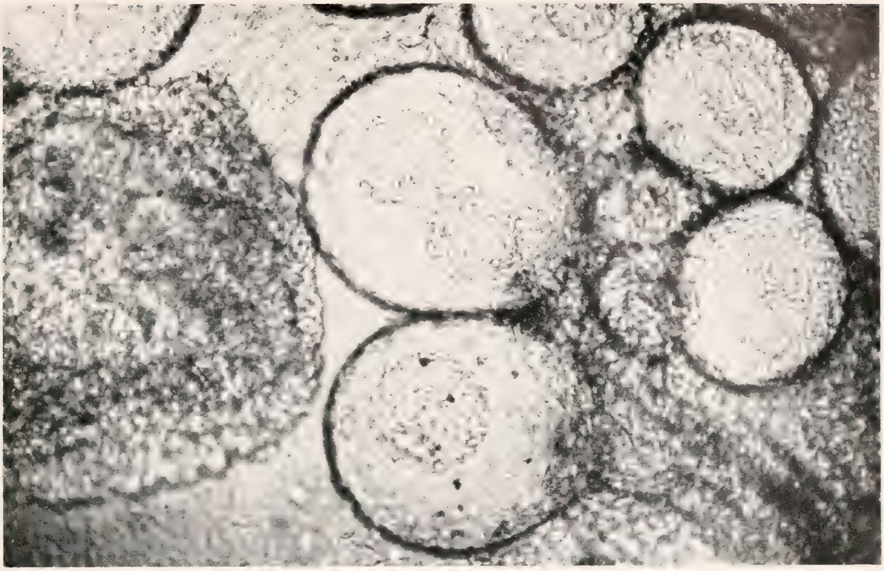

FIGURE 1. Binucleate #2 photographed *in situ* in unfixed ovarian tissue. Adjacent is the mononucleate used as a control for comparative measurements. Included also are younger oocytes and an older yolk-filled one. 430 ×

Measurements. Three different methods of measurement of egg and nucleus diameters were employed, depending on the exigencies of the moment.

Fixed eggs: When an egg with two nuclei was found, it was measured with an ocular micrometer in at least three or four diameters to correct for any variation from the spherical condition. For comparison as a "control" a single-nucleated cell of approximately the same size was located as close in the field as possible and its diameter measured. Similarly the nuclear diameters of both eggs were determined. From these data the volumes of the eggs and of their nuclei were calculated, assuming them to be spherical.

Fresh unfixed material was treated in one of two quite different ways: Usually the living binucleate was photographed *in situ,* together with a nearby mononucleate. In two cases both were fortunately in the same field. (For one, see Figure 1.) Without changing the setting of the microscope or camera, a stage micrometer was also photographed. The films were processed together and enlarged to the same magnification. Measurements were made by means of these photographs. Occasionally, when a camera was not available, camera lucida drawings were carefully made of the living binucleate, its control, and the stage micrometer, and measurements obtained from these drawings.

Extreme care was taken with all calibrations so that the measurements as finally presented in the tables are comparable with each other.

OBSERVATIONS

Fifty-six binucleate and two trinucleate eggs were found among the 249,616 small transparent primary oocytes observed. This total represented all the eggs with visible nuclei found in 25 frogs. Thus the binucleates constituted a percentage of 0.022% or a ratio of 1 to 4,457 ova.

The percentages of multinucleates in the frogs deviated significantly from one another. Fifteen of the 25 females possessed no multinucleated eggs at all. Of the 10 with multinucleated oocytes, 8 contained very few each, only one to four. However, in the ovaries of two individuals a large number of ova with two nuclei were found, namely 14 or 0.093% in one (#44), and 24 or 0.175% in the other (#39). This indicates that certain females are more prone to produce the multinucleate condition than others where it is determined by chance. This situation, while certainly of physiological significance, is not unusual. The literature abounds with similar cases. Indeed, Parmenter's (1952) rare diploid metaphases were found in three virgin eggs, all of which came from the same female, whereas none appeared in eggs from 11 other frogs. He discussed the literature in some detail (pp. 253–254).

There was considerable variation in the number of eggs found in each frog. The range was from 5,426 to 16,066. There did not appear to be any relationship between the abundance of ova in a female and her size as indicated by the length, measured from nose tip to anus.

Three of the 25 frogs contained ovaries with considerably more eggs than the others, 13,687–16,066. The two most productive of multinucleates were among these, but a frog with 13,717 eggs possessed only one binucleate. Moreover, the first three eggs which were found with two nuclei were in an individual with small ovaries containing a total of only 5,632 eggs.

One could not predict, therefore, from external conditions such as the size of the animal, or abundance of eggs, whether multinucleate oocytes were likely to be present.

The sizes of those oocytes containing either two or three nuclei varied in diameter from very small, 0.099 mm., to 0.350 mm. (Table I). Nine ova were smaller than 0.200 mm., *i.e.,* in "stages 1 or 2" (Duryee, 1950). The rest, including the two trinucleates, were all in "stage 3." The majority of the oocytes (20) were found to measure between 0.200 and 0.300 mm. Even in one female the multinucleated eggs varied markedly in size. In female #39 which produced the 24 binucleates the diameters of the oocytes ranged from 0.163 to 0.323 mm. with most from 0.200 to 0.300 mm. Also in frog #44 (14 bi- and 1 trinucleate) the variation included the tiniest egg of all (0.099 mm.) and extended to 0.292 mm.

It is recognized that within an ovary a condition of egg growth is in progress with the various stages of growth distributed indiscriminately throughout the structure. A suggestion of this is seen in Figure 1 where one can observe a yolk-filled egg of a later stage adjacent to the mono- and binucleates which were compared, and nearby much smaller eggs. It is thus easy to see how the oocytes to be compared were chosen. The senior author was much concerned with the possibility that, due to the extended growth period of primary oocytes, the cells compared, although of the same size, might not have been growing for the same length of time. This difference in the age of the oocytes would not affect the validity of the observations, merely the conjectures as to interpretation.

In an attempt to find a clue to the chromosome content of each nucleus, the nuclear volumes of the bi- or trinucleated cells were determined and compared with that of the nucleus of the normal mononucleate which would serve as a "con-

TABLE I

Occurrence of binucleate and trinucleate oocytes in frogs (Rana pipiens)

A. Binucleate oocytes

Binucleate oocyte number	Frog number	Nuclear volumes (mm.^{3} × 10^{-4})				Cell diameters (mm.)	
		Binucleates			Mono-nucleate control	Binucleate	Mono-nucleate control
		Nucleus a	Nucleus b	Sum of a and b			
Binucleates with the volume of both nuclei approximately equal							
1	36	4.94	4.94	9.88	12.3	0.350	0.334
2	36	5.24	5.24	10.5	10.2	0.275	0.260
3	36	6.83	6.83	13.7	10.8	0.303	0.291
8	39	14.2	13.0	27.2	36.0	0.339	0.343
12	39	7.05	6.28	13.3	17.7	0.290	0.290
13	39	5.11	5.72	10.8	7.59	0.215	0.220
14	39	10.4	8.64	19.0	15.3	0.289	0.266
15	39	7.27	6.59	13.9	11.9	0.263	0.265
18	39	2.35	2.04	4.39	3.79	0.162	0.161
19	39	7.16	7.43	14.6	14.0	0.272	0.271
21	39	3.97	4.18	8.15	6.74	0.209	0.208
23	39	5.89	5.89	14.2	15.7	0.280	0.256
24	39	6.59	7.59	14.2	11.5	0.239	0.234
27	39	5.89	5.75	11.6	11.5	0.248	0.276
28	39	10.0	12.5	22.5	23.5	0.323	0.321
29	42	3.79	4.08	7.87	6.09	0.191	0.190
31	44	5.59	5.36	11.0	7.59	0.215	0.225
35	44	1.75	1.65	3.40	3.52	0.137	0.137
36	44	9.14	8.04	17.2	13.1	0.258	0.247
37	44	0.662	0.593	1.26	1.75	0.099	0.102
38	44	5.02	5.46	10.5	21.7	0.292	0.305
39	44	2.86	3.17	6.03	7.00	0.193	0.199
41	44	3.62	4.49	8.11	11.9	0.256	0.251
44	44	3.52	3.52	7.04	5.11	0.183	0.177
45	44	1.03	0.98	2.01	1.81	0.114	0.113
48	48	3.42	4.49	7.91	9.32	0.207	0.215
49	48	7.01	6.89	13.9	15.8	0.245	0.245
51	51	6.09	5.02	11.1	9.07	0.229	0.223
57	59	0.95	0.95	1.90	2.35	0.119	0.120
Binucleates with markedly unequal volumes							
30	44	1.19	0.39	1.58	1.25	0.113	0.112
43	44	8.04	4.90	12.9	8.04	0.237	0.237

B. Trinucleate oocytes

Trinucleate oocyte number	Frog number	Nuclear volumes (mm^3 × 10^{-4})					Cell diameters (mm.)	
		Trinucleates				Mono-nucleate control	Tri-nucleate	Mono-nucleate control
		Nucleus a	Nucleus b	Nucleus c	Sum of a, b, and c			
1	44	5.96	6.09	6.34	18.4	15.1	0.271	0.266
2	51	3.01	4.37	4.26	11.6	9.32	0.212	0.214

trol." The sum of the volumes of the twin nuclei was approximately equal to that of the single one chosen for comparison in 29 measurable cells, and markedly unequal in 2 (Table IA). Four others possessed what appeared to be unequal nuclei, but due to misshapen cells or nuclei, or visible shrinkage in one or two cases, meaningful measurements seemed impossible. These were omitted from the table, as were three ova with two nuclei of apparently the same size where the conditions were such that the measurements were questionable. The other twenty eggs, although clearly binucleate, could not properly be measured.

In none of the binucleates did the volume of either of the two nuclei approach that of the selected "control," except in the case of egg #43. It will be seen from Table I that the larger nucleus had exactly the same volume as that of the mononucleate control, and the smaller was somewhat more than half that volume. One other difference between the two nuclei of egg #43 besides size should be mentioned. There is a conspicuous dissimilarity in the granular appearance. The smaller one resembles the usual binucleate in the peripheral arrangement of the large chromatic granules (nucleoli), whereas in the larger nucleus these bodies tend to be smaller and distributed more uniformly throughout the nucleoplasm.

The total volume of the twin nuclei that were of the same size approximately equalled that of the single nucleus of the cell chosen for comparison (Table IA). There was one exception also to this statement in the case of egg #38. In this interesting cell the sum of the volumes of the two nuclei was only about one-half the volume of the nucleus of the mononucleate. Measurements of additional control cells confirmed this relationship.

While searching for the cells with two nuclei, unexpectedly the two trinucleates were found. These conformed to the general pattern for binucleates in that the diameters of the cells were in the same size range, and the sum of the volumes of the three nuclei approximated that of a "control" mononucleate. Both were located in ovaries containing cells with two nuclei also. Trinucleate #1 was found in the same lobe as binucleate #43 which possessed the large nucleus equal in size to that of the control. The three nuclei of trinucleate #1 (Table IB) were of about the same size. Since in both cases the three nuclei were at different levels within the cell a photograph was not feasible. In trinucleate #2 two of the nuclei were larger in size than the other, and equal in volume to each other. Interestingly, a similar difference in granular appearance existed between the two large nuclei and the smallest one as was described for binucleate #43. The small nucleus had fewer and larger chromatic granules whereas the two larger nuclei possessed smaller and more numerous ones.

DISCUSSION

The following ideas were found among Doctor Parmenter's notes. No further discussion will be attempted.

The unequal-sized binucleates and the trinucleates suggest that a possible origin may have been from separated groups of chromosome vesicles. But such an origin does not seem probable for the large proportion of binucleated oocytes, the nuclei of which were of equal volume. More likely this condition arose by a nuclear division followed by a failure of cytosomic division. These binucleated cells may have been produced during the last oogonial division.

Should further information demonstrate that in some cases each of the two equal-sized nuclei contains the full set of 13 tetrads, both the normal behavior and the possible failure of one of these nuclei to give off its polar body in either meiotic division would produce various chromosome numbers in mature eggs and in embryos resulting either from parthenogenetic stimulation or from fertilization.

LITERATURE

Information in the literature concerning multinucleate oocytes is sparse. Indeed Humphries (1956) in discussing the origin of spontaneous polyploidy in *Triturus viridescens* hesitated to "assume" (p. 120) the existence of a binucleate oocyte as a source of diploidy in such embryos. He preferred the explanation of (p. 120) "direct pathways actually seen to exist," one of which he describes in his paper on the effects of heat shock on the first meiotic division. We have now shown that binucleate primary oocytes actually do exist, at least in *Rana pipiens*. Beatty (1957) who reviewed the literature on polynuclear ovarian eggs, states that such are (p. 81) "rare but widely spread." He mentions twelve species of mammals (including man), reptiles, birds, insects, but no amphibians. Sentein (1958) was able to produce multinucleated eggs of *Triturus* and *Pleurodeles* by treatment with phenol. He saw cytoplasmic division inhibited. This treatment constitutes, of course, an unnatural source of the polynucleate condition.

In embryonic tissue Parmenter (1937, 1940) reported multinucleated erythrocytes in parthenogenetic frog larvae, also five epithelial cells each with two nuclei plus one with three. Moore (1957) has presented evidence that chromosomal vesicles constitute a basis for the origin of what she refers to as "double nuclei" (p. 209) in early embryos of diploid frog hybrids. She reviewed the literature extensively. Of interest here is her discussion of the distribution of peripheral coarse vs. diffuse fine chromatin in some of the cell nuclei of her material. She states that similar conditions were also found by King and Briggs, by Brachet, and by others. She wonders if some of the (p. 222) "so-called nuclear anomalies" found in hybrids are not really of (p. 222) "normal occurrence in the development of amphibian eggs."

The occurrence of the bi- and multinucleate condition *normally* in liver tissue of mammals including human is well known and has been reviewed recently by Inamdar (1958). By microspectrophotometric measurements of DNA in resting nuclei of mouse liver, he was able to confirm the conclusions of Beams and King (1942) and others that the origin of the binucleates is best explained by division of the nucleus without division of the cell.

No discussion will be undertaken on the often-noted polynucleate condition in tissue cultures, or in pathological material.

SUMMARY

1. Fifty-six binucleated and two trinucleated cells were found among 249,616 young transparent primary oocytes in post-ovulation ovaries of 25 females of *Rana pipiens* (0.022%).

2. Multinucleated oocytes were absent in 15 females, present in 10. Eight of these 10 produced only one to four binucleates each; one female was the source

of 1 tri- and 14 binucleates; and another gave the remarkable number of 24 (0.175%), all binucleates.

3. The multiple nucleated condition did not seem to be correlated in any way with the size of the female, the abundance of her eggs, nor the size of the egg.

4. In none of the binucleates did the volume of either of the two nuclei approach that of the nucleus of a mononucleate of the same size. The one exception was egg #43 where one nucleus did have exactly the volume of the control, the other about half that.

In 29 of the 31 measurable binucleates the two nuclei were of approximately the same size and the sum of the two volumes equalled that of the mononucleate, except in one case where it was half.

The origin of binucleate oocytes remains uncertain; it may be connected with a final division of an oogonial nucleus that was not followed by cell division. In this case the two nuclei would both be diploid.

5. The two trinucleates conformed in general to the same pattern as the binucleates as to their distribution, size of oocyte, and the volumes of their nuclei. The sum of the three nuclear volumes approximated that of the mononucleate.

6. In two cases where the nuclei were markedly unequal in size, there was a definite difference in the appearance of their chromatic granules. These bodies were more abundant and finer in the larger nuclei, peripheral, larger and more distinct in the smaller nucleus.

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COLD DEATH IN THE GUPPY[1]

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There are numerous reports describing the death of vast numbers of fish subsequent to cold waves, often where the water temperature remained well above 0° C. (Wilcox, 1887; Verrill, 1901; Vatova, 1929; Storey and Gudger, 1936; Storey, 1937; Miller, 1940; Gunter, 1941; Gunter, 1947; Mosevich, 1944).

Maurel and Lagriffe (1899) divided the changes induced by placing fish into colder and colder water into five stages. Stage five consisted of convulsions, loss of equilibrium, complete paralysis, and a state of apparent death; the fish would revive only if warmed within a few minutes. If not warmed, they would go on to die without showing any visible signs of life. Doudoroff (1942) called this "primary chill coma."

At less extreme temperatures a different sequence of events occurred. The fish would recover from the initial shock, resume respiration, if it had stopped, and take on a relatively normal appearance. If kept at such a temperature for some hours or days, the fish would show increasing distress and finally cease to respond to stimuli and to respire. This characteristic response to less extreme temperatures, Doudoroff (1942) called "secondary chill coma." In "primary chill coma" the fish die without a return of vital signs, unless rewarmed; in "secondary chill coma," vital signs, if they disappear, return for a time and the fish then gradually die unless rewarmed.

Virtually nothing is known of the mechanism of death due to "primary chill coma." Brett (1952) experimented with cold tolerance in young Pacific salmon and concluded that death occurring in the first hour of a cold exposure was probably due to a disturbance of the central nervous system. It has been shown that with decreased acclimation temperature a lower temperature is required to induce "primary chill coma" (Samochvalova, 1938) and the fish can withstand longer exposures to a lethal cold temperature (Fry, Brett and Clawson, 1942; Doudoroff, 1942; Brett, 1956).

Only Doudoroff (1945) has proposed a mechanism of death due to "secondary chill coma." He showed that the death of *Fundulus*, a salt-water fish, at slowly lethal temperatures in sea water was preceded by osmotic dehydration of tissues, and was delayed in diluted sea water. He concluded that osmoregulative failure was one of the causes of slow death in cold sea water.

The characteristics of cold tolerance in the guppy, *Lebistes reticulatus*, have never been thoroughly explored. The guppy is an ideal subject for inquiry into

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the effects of cold on fish. It is hardy, inexpensive, and easily procured. More than 4500 guppies were used in the past three and one-half years to secure the information presented in this paper.

This paper will propose for the first time a possible mechanism of death due to "primary chill coma" in fish.

METHODS AND MATERIAL

Approximately 1000 guppies were obtained from aquatic plant tubs in the greenhouses of the University of Pennsylvania, Department of Botany. The remainder were bought from Florida fish farms. The fish were acclimated for two weeks to five-, ten-, or fifteen-gallon stock aquaria, with up to 40 fish per gallon of water. The fish were fed various commercial brands of dry fish food and Dash dog food once daily.

Randomly chosen fish were taken from stock aquaria by means of a soft net and quickly placed into the test water which was maintained at uniform temperature (0.1° C.) by gentle shaking of the container unless otherwise stated, by the gradual addition of ice to a constant temperature bath into which the test container was immersed. Temperatures were read from standardized mercury thermometers graduated to 0.1° C. or 0.05° C. At the completion of a test exposure or a series of stepwise test exposures the fish were quickly removed and placed in water from the stock tank.

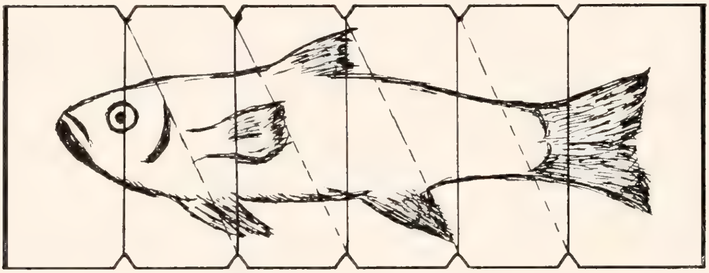

FIGURE 1. Comatose fish secured to a notched glass slide with a soft cotton thread.

Once a fish regained normal respiration and was able to swim normally after a test exposure, it almost invariably lived. Those fish alive 24 hours after test exposures were considered as having survived the insult. Deaths after this 24-hour period were few, always less than 5%, and seemed to depend mostly on the general condition of the fish prior to the cold exposure.

In the experiments in which fish were subjected to cold in oxygen-depleted water or oxygen-enriched water, the water was vigorously bubbled and equilibrated with 100% nitrogen or 100% oxygen for one hour immediately prior to its use. In approximately one-half of these experiments a sample of test water was siphoned directly into a sampling jar at the midpoint of the cold exposure and immediately subjected to a Winkler oxygen determination. In all the experiments where a Winkler oxygen determination was performed, distilled water was used in the test container, rather than stock tank water, so as to minimize the amount of organic material which might interfere with the accuracy of the oxygen determination. The substitution of distilled water for stock tank water did not alter the results. In those experiments where an oxygen determination was not performed, the oxygen content was estimated and rounded off to the nearest 5 mg./L.

Some of the data to be presented are observations on individual fish of the relationship of respiration to the presence of a functioning circulation. Gill and mouth respiratory movements were easily seen with the naked eye. The criterion for the presence of a functioning circulation was the presence of blood flow in the arteries and veins at the base of the fish's tail. Thirty-five or 100 diameters magnification of these vessels rendered blood flow clearly visible. A fish after being paralyzed in the cold test water was quickly placed on a notched glass slide and gently secured with soft cotton thread as diagrammed in Figure 1. This necessitated removing the fish from the test water for only a few seconds. The slide was replaced into the test container on a microscope stage and the tail vessels brought into focus. The time intervals required for respiration and circulation, respectively, to stop after chilling and to start after warming, were measured by means of a stop-watch. The microscope and other equipment were set up in a constant temperature cold room set at the temperature of the test water. The temperature of the test water remained constant to within 0.2° C. during each experiment.

All data were analyzed statistically by a special method of computing chi square in an Rx2 table (Snedecor, 1956, p. 227).

RESULTS

The guppy's normal temperature range, according to Innes (1955), is 16° C. to 40° C.; Gibson (1954), however limited the upper temperature to 32° C. When a guppy was exposed suddenly to water below 10° C., it exhibited the characteristics of "primary chill coma." Often after a few minutes at the low temperature, the fish's chromatophores expanded, causing the fish's color to darken. Exposures to cold temperatures above 10° C. resulted in "secondary chill coma."

Upon being replaced in a stock tank after "primary chill coma," the fish would usually begin to respire within eight minutes and almost invariably within twenty minutes, if they were to survive. Occasionally a fish would revive, show respiratory movements for several minutes to a few hours and then die. In "secondary chill coma," if a fish had gradually ceased to respire during the cold exposure, it usually did not revive when replaced in stock water of normal temperature, but if it was still respiring when taken from the cold, it usually survived.

I. Primary Chill Coma

A. Study of a single population

Approximately 700 guppies of all sizes were taken from a large aquatic plant tub in a University of Pennsylvania Botany Department greenhouse and acclimated to 23° C. ± 1° C. for 10 days. Batches of 10 adults, 5 males (80–130 mg.) and 5 females (100–900 mg.), and batches of 10 young guppies (6–18 mg.) were randomly picked and subjected to specific cold exposures. The rest of the fish were then acclimated to 30° C. ± 1° C. for 10 days; batches

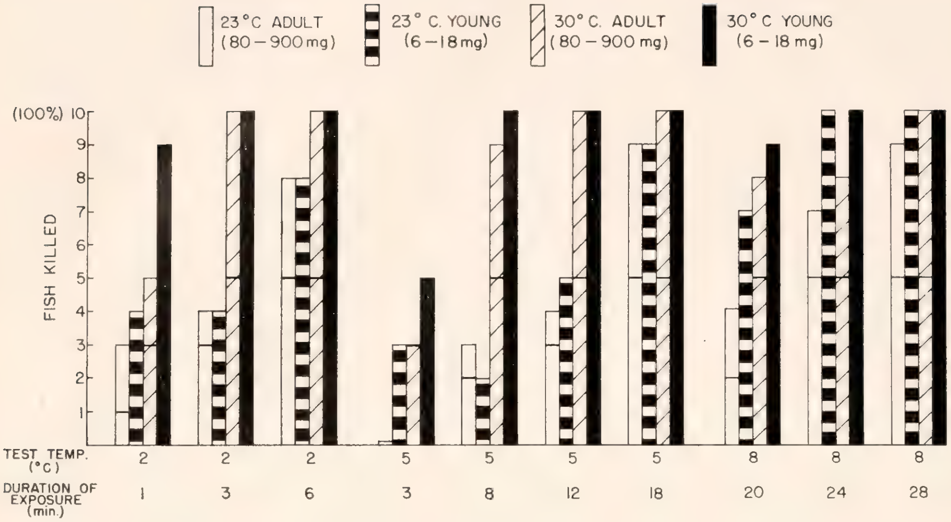

FIGURE 2. Death due to "primary chill coma" in the guppy. The black bar separates the females above from the males below.

of these adults and young were then subjected to the same cold exposures as those fish acclimated to 23° C. The results are summarized in Figures 2 and 5; Figure 2 shows the results of exposures to temperatures causing primary chill coma, and Figure 5 the results of exposures to temperatures causing secondary chill coma.

1. Effect of the temperature, duration of cold exposure, sex, size, and acclimation temperature on mortality

Combining all the data in Figure 2 it is seen that: (1) the lower the temperature, the more rapidly lethal effects occurred; (2) each increase in

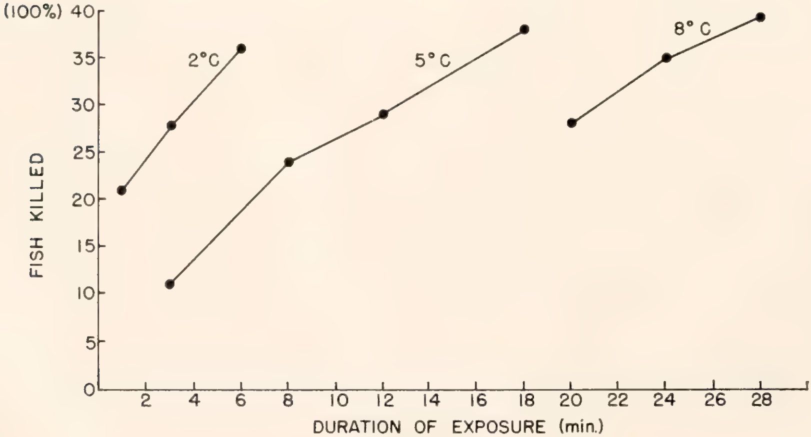

FIGURE 3. Total number of fish killed by each cold exposure at 2° C. ($p < 0.005$), 5° C. ($p << 0.001$), and 8° C. ($p < 0.005$).

the duration of exposure to a specific cold temperature was associated with an increase in the total number killed (Fig. 3); (3) males are less cold-tolerant than females ($p < 0.005$); (4) young are less cold-tolerant than adults ($p = 0.005$); (5) in both young ($p < 0.001$) and adults ($p < 0.001$) tolerance to "primary chill coma" is inversely related to acclimation temperature.

2. Effect of acclimation temperature on tolerance to anoxia at normal temperatures

When placed in 21° C. oxygen-depleted water (less than 0.25 mg. O$_2$/L.) for 15 minutes, none out of 20 fish acclimated to 23° C. succumbed, yet 10 out of 20 of fish acclimated to 30° C. died ($p = 0.001$). Tolerance to oxygen lack is thus inversely proportional to acclimation temperature.

Sumner and Doudoroff (1938), using boiled sea water and 0.001 molar potassium cyanide in sea water, likewise demonstrated that in the gobie, *Gillichthys mirabilis* Cooper, tolerance to anoxia was inversely related to acclimation temperature.

B. Lethal roles of the rapidness and repetition of chilling

1. Rapid versus slow chilling

In experiments where the water temperature was gradually reduced from the acclimation temperature to 2° C. in 15 minutes or to 8° C. in 7 minutes, 13 out of 16 fish died, whereas with rapid chilling to the same temperatures 13 out of 16 fish also died.

2. Lethal role of repetitive chilling

A batch of 20 adult guppies, 10 males and 10 females, acclimated to 23° C. was placed at 5° C. for one minute and subsequently at 23° C. for

TABLE I

Relationship of circulatory and respiratory functions in death due to "primary chill coma" in the guppy. Fish were acclimated to 29° C. for ten days

Fish No.		When chilled		When placed at 25° C.		Resp. started at	Condition 24 hrs. later
		Resp. stopped at	Circ. stopped at	Circ. started at	Circ. stopped at		
#1	5° C. for 3 min. (½ LD50)	less than 10 sec.	30 to 80 sec.				Alive
#2	5° C. for 3 min. (½ LD50)	less than 10 sec.	60 sec.				Alive
#3	5° C. for 5 min. (1 LD50)	3.6 sec.		15 sec.		75 sec.	Alive
#4	5° C. for 5 min. (1 LD50)	4.7 sec.		10 sec.		50 sec.	Alive
#5	2° C. for 3 min. (3 LD50)	2.3 sec.		15 sec.	2 to 11 min.	—	Dead
#6	2° C. for 10 min. (10 LD50)	3.5 sec.		210 sec.	7 to 10 min.	—	Dead
#7	2° C. for 65 min. (65 LD50)	2.6 sec.		—		—	Dead
#8	2° C. for 75 min. (75 LD50)	4.0 sec.		—		—	Dead

one minute. These exposures were repeated six times in succession. Each time the fish were chilled they lost all respiratory and swimming movement; but when they were placed at 23° C., most of them regained respiratory movements. None of these fish died.

C. Relationship of respiratory movements to the presence of a functioning circulation (Table I).

Careful individual observations were made on 30 fish subjected to 2° C., 5° C., or 8° C. Twenty fish received an LD50 exposure, 8 received 3 to 12 times the LD50 exposure, and 2 received over 60 times the LD50 exposure. In each of these 30 fish a complete cessation of all respiratory and swimming movements occurred within 10 seconds. The results were consistent; the data from 8 typical fish of this group are presented in Table I. The circulation, as judged by blood flow in the tail vessels of two fish (Fish #1 and #2, Table I), ceased approximately one minute after the fish were placed at 5° C.

If the heart beat returns it does so before there are any respiratory movements, as described by Britton (1924); in the present experiments if the circulation returned it did so before the respiratory movements, and the respiratory movements never returned unless a functioning circulation was already present.

Fish #3 and #4 (Table I) were subjected to an LD50 cold exposure and only transient respiratory and circulatory depression resulted. In all of the 20 fish subjected to an LD50 cold exposure the circulation returned. Respiration returned in 13 out of the 20 and they survived; in 4 others out of the 20, respiration returned for a brief period, subsequently stopped and the fish died; in the 3 others remaining out of the 20, respiration did not return and they died.

Fish #5 and #6 (Table I) were exposed to cold long enough to permit permanent respiratory depression but only transient circulatory depression. This resulted in the 8 fish exposed to 3 to 12 times the LD50 cold exposure as well as 3 of the 20 fish which received approximately an LD50 cold exposure.

Only 2 of the 30 fish (#7 and #8, Table I) received cold exposures of greater than 12 times the LD50 exposure. Neither the circulation nor the respiration returned in either fish and they of course did not survive.

D. Effect of increasing the tonicity of the chilling water

Fish were chilled at 5° C. for 8 minutes in distilled water, 0.32 molar sodium chloride (F.P. − 1.2° C.), or 0.65 molar glucose (F.P. − 1.2° C.) (the freezing point of teleost body fluid is between − 0.5° C. and − 0.9° C.; Brett, 1956). Increasing the tonicity of the chilling water exerted no significant effect on the guppy's cold tolerance, as shown by the fact that 21 out of 30 fish chilled in distilled water, 21 out of 30 fish chilled in 0.32 molar sodium chloride, and 20 out of 30 fish chilled in 0.65 molar glucose succumbed.

E. Effect of varying the gaseous content of the test water

1. Effect on mortality of varying the oxygen content and carbon dioxide content of the chilling water (Table II)

Increased $O_2 + CO_2$ or increased O_2 alone, during a cold exposure, lessened the lethality of the exposure (Exps. #1 and #3 Table II). Increased CO_2 alone had no such effect (Exp. #2 Table II). Exp. #4 (Table II) demonstrated that decreased O_2 during a cold exposure increases the lethality of the exposure.

TABLE II

Effect of increased oxygen and carbon dioxide (Exp. #1), increased carbon dioxide (Exp. #2), increased oxygen (Exp. #3), and decreased oxygen (Exp. #4) during cold exposures on tolerance to "primary chill coma"

	Treatment	Test water equilibrated with	O_2 content mg./L.	Number killed	Level of significance
Exp. #1	5° C. for 8 min.	Air 95% O_2 + 5% CO_2	14.1 51.1	49 of 59 (83%) 8 of 55 (15%)	$p \ll 0.001$
Exp. #2	5° C. for 10 min.	Air 21% O_2 + 5% CO_2	14 14	39 of 40 (98%) 38 of 40 (95%)	$p = 0.7$
Exp. #3	5° C. for 10 min.	Air 100% O_2	13.6 51.5	49 of 50 (98%) 31 of 46 (67%)	$p \ll 0.001$
Exp. #4	8° C. for 10 min.	Air 100% N_2	13.3 <0.25	6 of 40 (15%) 15 to 40 (38%)	$p = 0.02$

2. Effect on mortality of increased oxygen before, during, or after a cold exposure (Table III)

Increased environmental oxygen during or after a cold exposure decreased the lethality of the exposure, but increased environmental oxygen before the exposure did not significantly alter this lethality (Table III). There is no significant difference ($p = 0.1$) between increased oxygen during or after a cold exposure (Batches #3 and #4, Table III).

3. Effect of increased oxygen on mortality when given during or after exposures to 2° C.

Increased oxygen during one-minute cold exposures to 2° C. did not lessen mortality significantly ($p = 0.2$); 20 out of 30 young guppies (6–18 mg.) died when chilled in oxygen-depleted water ($<$ 0.30 mg. O_2/L.), and 20 out of 40 died in O_2-enriched water (50 mg. O_2/L.).

Twenty minutes in oxygen-enriched water after a three-minute exposure to 2° C. lessened mortality significantly ($p = 0.005$), for 24 out of 25 adult guppies died when replaced in a stock tank (10 mg. O_2/L.) while only 8

TABLE III

Effect of increased oxygen before, during, or after a cold exposure. Batches of 24 young fish (8 to 20 mg.) were chilled at 5° C. for 8 minutes. Batches #1, #2, and #4 were chilled in oxygen-depleted water (less than 0.30 mg. O_2/L.). Batch #3 was chilled in oxygen-enriched water (50 mg. O_2/L.). (Batch #2 was placed in oxygen-enriched water 40 mg. O_2/L.) for 20 minutes prior to the cold exposure and batch #4 for 20 minutes after the cold exposure.

Batch No.	Treatment	Per cent killed	p Value as compared to the control
1	CONTROL (Normal O_2 before and after)	100	
2	Increased O_2 BEFORE cold exposure	96	0.4
3	Increased O_2 DURING cold exposure	21	<0.001
4	Increased O_2 AFTER cold exposure	46	0.001

out of 25 died when they were placed in oxygen-enriched water (40 mg. O_2/L.) of normal temperature prior to return to the stock tank.

F. Significance of anoxia and cold in causing death

1. Tolerance to anoxia at normal temperatures

Three randomly chosen batches of 10 adult guppies each, acclimated to 27 ± 2° C., were placed in oxygen-depleted water at 26° C. (< 0.30 mg. O_2/L.) for different periods of time. Eight minutes of exposure killed none; 15 minutes killed 50%; and 20 minutes killed 100% ($p < 0.001$).

2. Interaction of cold and anoxia (Table IV)

There is a statistically significant difference in number killed between batch #3 and batches #1, #2, and #4 (Table IV). Nine minutes of anoxia prior to 5° C. for one minute (#3) killed significantly more than 5° C. for one minute alone (#1) ($p = 0.02$), 10 minutes of anoxia alone (#2) ($p = 0.005$), or 9 minutes of anoxia after 5° C. for one minute (#4) ($p = 0.05$). This indicated that anoxia before a cold exposure is more lethal than anoxia after a cold exposure.

TABLE IV

Interaction of cold and anoxia in producing death in "primary chill coma." Fish acclimated to 23 ± 1.0° C. for ten days

Batch No.	Treatment	Number killed
	Decreased oxygen (<0.25 mg./L.)	
1	5° C. for 1 min.	2 of 20 (10%)
2	25° C. for 10 min.	1 of 20 (5%)
3	25° C. for 9 min. followed by 5° C for 1 min.	9 of 20 (45%)
4	5° C. for 1 min. followed by 25° C. for 9 min.	3 of 20 (15%)
5	5° C. for 10 min.	18 of 20 (90%)
	Increased oxygen (50 mg./L.)	
6	5° C. for 10 min.	2 of 20 (10%)

When batch #5 (Table IV) was exposed to 5° C. for 10 minutes, a significantly higher number ($p = 0.005$) died than in batch #3 where fish were exposed to anoxia for 9 minutes and then to 5° C. for one minute. There is a highly significant difference between batch #5 and all the other batches. Batch #6 was exposed to 5° C. for 10 minutes in oxygen-enriched water (50 mg. O_2/L.); but only 2 out of 20 died as compared to 18 out of 20 in batch #5 which was exposed to 5° C. for 10 minutes in oxygen-depleted water (< 0.25 mg. O_2/L.) ($p \ll 0.001$).

G. Effect of prior anesthesia with 1% urethan (Fig. 4)

Prior anesthesia with 1% urethan decreases the lethal effect of "primary chill coma" ($p = 0.005$) (Fig. 4).

H. Effect of varying the sequence of two cold exposures (Table V)

Comparing batches #1 and #2 (Table V), a significantly higher number died ($p = 0.001$) among the fish exposed to the colder temperature first.

A suggestive decrease in mortality ($p = 0.1$) to an exposure to 2° C. for one minute occurred if the fish were first exposed to 8° C. for 4 minutes (Batches #2 and #3, Table V).

There is no statistically significant difference ($p = 0.25$) between 2° C. for

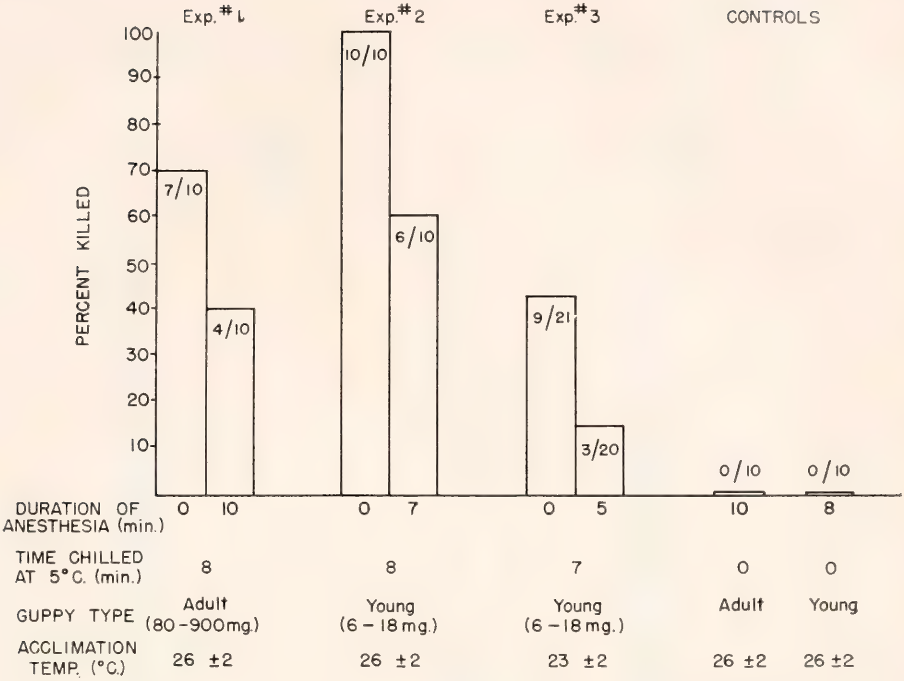

FIGURE 4. Protective effect of anesthesia with 1% urethan immediately before a cold exposure ($p = 0.005$).

5 minutes (Batch #4), and 2° C. for 1 minute when followed by 8° C. for 4 minutes (Batch #1).

There is a profound difference ($p \ll 0.001$) in lethal effect between 2° C. for 5 minutes (Batch #4), and 8° C. for 5 minutes (Batch #5).

II. Secondary Chill Coma

A. Study of a single population (Fig. 5)

1. Effect of the duration of a cold exposure on mortality

At 10.5° C., 20 out of 40 fish exposed for one hour died while 29 out of 40 exposed for one and one quarter hours died ($p = 0.05$). Only two

TABLE V

Effect of varying the sequence of two cold exposures. Three groups of ten adult guppies were used for each treatment

Batch No.	Treatment	Number killed
1	2° C. for 1 min. followed by 8° C. for 4 min.	25 of 30 (83%)
2	8° C. for 4 min. followed by 2° C. for 1 min.	12 of 30 (40%)
3	2° C. for 1 min.	19 of 30 (63%)
4	2° C. for 5 min.	30 of 30 (100%)
5	8° C. for 5 min.	2 of 30 (7%)

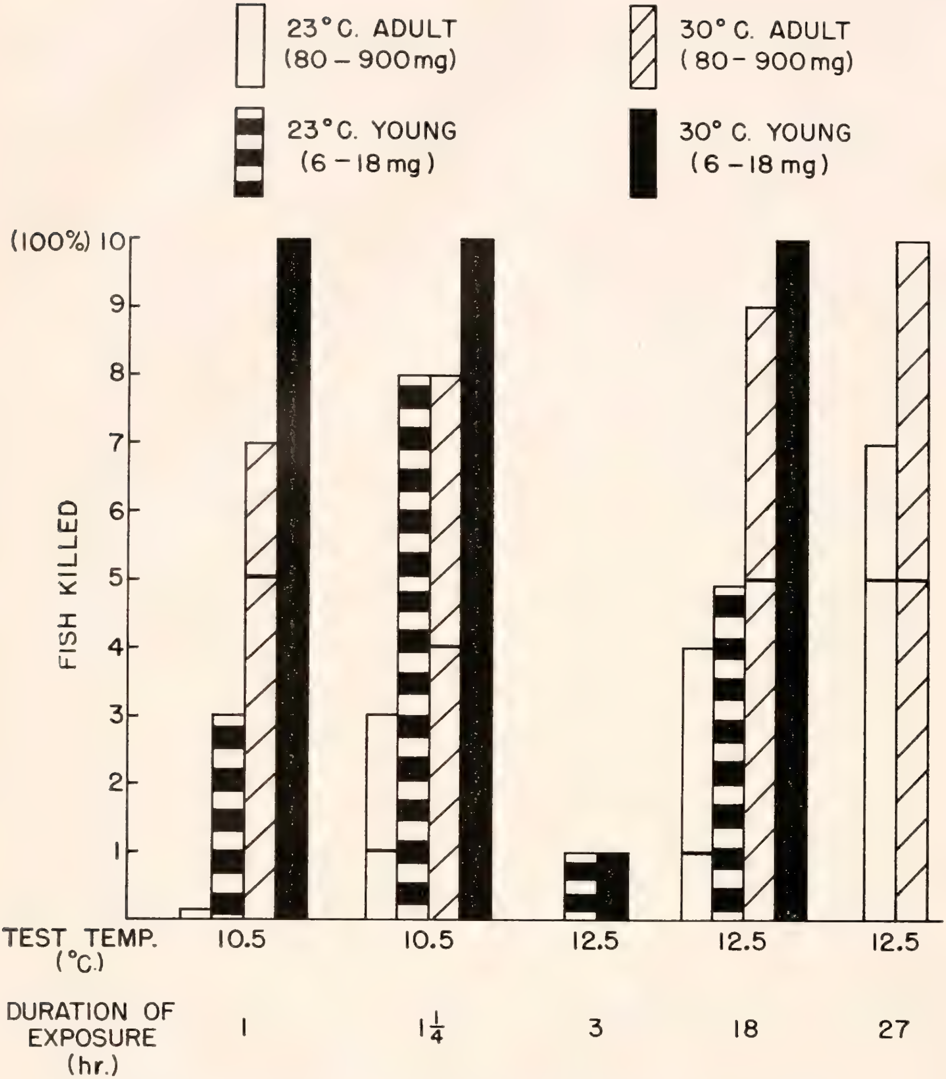

FIGURE 5. Death due to "secondary chill coma" in the guppy. The black bar separates females above from males below.

out of 20 young guppies exposed to 12.5° C. for 3 hours died while 15 out of 20 exposed for 18 hours died ($p < 0.001$). Thirteen out of 20 adults exposed to 12.5° C. for 18 hours died while 17 out of 20 exposed for 27 hours died ($p = 0.25$). (This last comparison is the only one of the three where the difference is not statistically significant.) These data indicate that in "secondary chill coma," as in "primary chill coma," the duration of exposure determines the mortality.

TABLE VI

Effect of increased oxygen content of the chilling water on tolerance to "secondary chill coma." Fish acclimated to 27 ± 2° C. Combining the results from the three experiments, $p = 1.0$

	Treatment	Number killed	
		10 mg. O_2/L.	45 mg. O_2/L.
Adult fish (100–800 mg.)			
Exp. #1	11.4 ± 0.3° C. for 15.5 hrs.	24 of 25 (96%)	25 of 25 (100%)
Exp. #2	12.1 ± 0.2° C. for 8.0 hrs.	15 of 20 (75%)	15 of 20 (75%)
Juvenile fish (20–40 mg.)			
Exp. #3	12.1 ± 0.2° C. for 12.0 hrs.	20 of 25 (80%)	19 of 25 (76%)

2. Effect of sex, size, and acclimation temperature on mortality

Comparing data in Figure 5, males do not seem less cold-tolerant than females ($p = 0.3$); but young are much less cold-tolerant than adults ($p = 0.005$). Cold-tolerance is inversely related to the acclimation temperature in both young ($p < 0.001$) and adults ($p = 0.005$).

B. Effect of increased oxygen during the cold exposure (Table VI)

Increased oxygen during secondary chill coma did not lessen mortality (Table VI).

C. Effect of isosmotic chilling medium on mortality

Two batches of young guppies (8–18 mg.) were subjected to 12.0° C. for 5.5 hours. Batch #1 was chilled in distilled water and batch #2 in 0.16 molar sodium chloride (F.P. − 0.6° C. This is approximately isotonic with teleost body fluids (Brett, 1956). Nine out of 23 fish in batch #1 succumbed; 10 out of 24 in batch #2 died. There is no significant difference between these two batches ($p = 0.75$).

DISCUSSION

Among the possible causes of death inherent in cold exposure, two factors may be excluded. The suddeness of a cold exposure is not of itself lethal, for sudden exposures did not cause more mortality than gradual exposures (IB1)[3] (Britton, 1924). Moreover, the cooling process *per se* is not lethal since even repetitive chilling into "primary chill coma" caused no mortality (IB2). At a specific cold temperature, the duration of cold exposure is the decisive determinant of lethality rather than the abruptness or repetition of the temperature change.

"Primary Chill Coma"

Taken altogether, the data suggest that "primary chill coma" kills by causing anoxic damage to a cold-depressed respiratory center. The permanent re-establishment of respiration after "primary chill coma" is the critical event in determining survival (IC). Even after prolonged lethal exposures to cold, the fish's circulation often returns within a few minutes (IC) and then, depending on one major variable, permanent respiration does or does not return. This variable is the amount of oxygen which the circulation can bring to the respiratory center to forestall anoxia damage while the cold depression of respiration subsides.

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[3] This refers to pertinent experimental data listed under results.

There appear to be several factors limiting the availability of oxygen. Sumner and Doudoroff (1938) showed that fish have a considerable oxygen reserve in their tissues. Consequently, in the present experiments the relative depletion or saturation of the fish's oxygen reserve partially determines the amount of oxygen that the circulation can carry to the fish's respiratory center. Normally the oxygen reserve is probably maximal, since increased oxygen before "primary chill coma" did not lessen mortality (IE2). If the oxygen reserve had been submaximal, then the extra oxygen should have diffused into the fish's superficial tissues (Privol'nev, 1956; Breder, 1941) and protected the animal against anoxia. Any process which depletes the oxygen reserve increases mortality. The oxygen reserve may be depleted by a prior anoxic period (IF2), decreased environmental oxygen during a cold exposure (IE1), or the extra oxygen consumption which accompanies the initial convulsive paroxysm. Anesthesia prior to a cold exposure lowered mortality (IG), probably by lessening the initial convulsive paroxysm and so lessening this initial depletion of the oxygen reserve.

The protective value of increased oxygen during and after chilling (IE) may be explained by the hypothesis that when the fish's oxygen reserve is depleted, more oxygen can diffuse into the fish and replenish that portion of the depleted oxygen reserve located in the cutaneous and subcutaneous tissues. Ultimately the oxygen probably protects by acting centrally. Nervous tissue is the most sensitive to anoxia of any vital tissue, and so it is probable that the increased oxygen benefits the respiratory center directly. This effect of increased environmental oxygen in promoting respiratory return and survival does not seem to be related to any reflex mechanism for at least two reasons: (1) Increased environmental oxygen would lessen rather than increase reflex stimulation of the respiratory center; (2) increased oxygen protected even when given only during the cold exposure and not when respiration actually restarted (IE).

The observation that four of the thirty individually observed fish died after respiration was re-established (IC) may have either of two alternative explanations: (1) There is an additional mechanism or mechanisms for death due to primary chill coma; (2) when rewarmed the cold depression of the respiratory center was relieved and it began functioning while still anoxic; this rendered it more anoxic and permitted irreversible damage to occur.

In experiments where oxygen was unaltered, mortality was increased by lowering the temperature of a cold exposure of a specific duration (IA1) (IH) and also by subjecting fish first to the lower of two sequential cold exposures (IH). The lower chilling temperature probably induces a more profound respiratory depression and a more vigorous initial convulsive paroxysm which more completely depletes the oxygen reserve.

Tolerance to "primary chill coma" is inversely related to acclimation temperature (IA1). Perhaps a higher acclimation temperature lessens the fish's oxygen reserve since there is less oxygen dissolved in body water of higher temperatures; however, this is partially offset by an increase in diffusion (Krogh, 1919). A higher acclimation temperature increases the overall metabolic rate so that the fish's oxygen reserve is depleted more rapidly. With an increase in the acclimation temperature the central nervous system would have an increased oxygen need (Freeman, 1950) and the respiratory center would become anoxic more rapidly.

Oxygen may be important also in the observations that young are less tolerant to "primary chill coma" than adults (IA1) and males are less tolerant than females (IA1). These less tolerant groups are smaller, have a proportionately higher metabolic rate (Muller, 1942), and so upon chilling might become anoxic more rapidly. Perhaps they would experience also a more profound respiratory depression, for their smaller size would permit the cold to penetrate more rapidly to the respiratory center. Their smaller size should permit a more rapid replenishment of oxygen reserve via cutaneous diffusion, but evidently this advantage is overshadowed by the higher metabolic rate and perhaps by the more rapid penetration of the cold in the smaller fish. It is possible that young guppies and or male guppies may have a respiratory center which is innately more susceptible to cold depression or anoxic damage or both.

"Secondary Chill Coma"

The experimental data presented in this paper give no indication of the cause of death due to "secondary chill coma." Osmoregulative failure (Doudoroff, 1945) does not seem to occur in the guppy, for chilling in isosmotic sodium chloride had no protective effect (IIC). Although increased oxygen protects against "primary chill coma," it does not protect against "secondary chill coma" (IIB).

Certain observations are perhaps significant. Fish acclimated to higher temperatures are more sensitive to "secondary chill coma" (IIA2); young are more sensitive than adults (IIA2). These more sensitive groups have a significantly higher metabolic rate and would exhaust their energy stores more rapidly so that death due to "secondary chill coma" might be some sort of exhaustion phenomenon. There does not seem to be a sexual difference in sensitivity to "secondary chill coma." The difference in metabolic rate between male and female guppies is perhaps not enough to produce significant differences in the limited number of fish used.

Despite the present inability to explain the mechanism or mechanisms of death due to "secondary chill coma," all the data that have been obtained to the present time can be reconciled with the proposition that death due to "primary chill coma" is caused by anoxic damage to a cold-depressed respiratory center.

I am indebted to the late Dr. L. V. Heilbrunn for first stimulating my interest in this problem and for providing laboratory space. I wish to thank Dr. John R. Brobeck for sponsoring this project and for his many valuable suggestions and encouragement, and Dr. Harold T. Hammel for his advice and aid.

SUMMARY

A. Observations concerning both "primary chill coma" and "secondary chill coma"

1. In guppies acclimated to 23 to 30° C., exposures to temperatures below 10° C. produced "primary chill coma" while exposures to lethal temperatures above 10° C. caused "secondary chill coma."

2. The duration of a cold exposure at a specific temperature is the decisive lethal

determinant rather than the chilling temperature *per se*. An increase in the duration of a cold exposure causes an increase in mortality.

3. Tolerance to a cold exposure is inversely related to acclimation temperature. Tolerance to oxygen lack at normal temperatures also is inversely related to acclimation temperature.

4. Males are less cold-tolerant than females in the temperature range of "primary chill coma." There does not seem to be a sexual difference in cold tolerance in the temperature range of "secondary chill coma."

5. Young guppies (6–18 mg.) are less cold-tolerant than adults (80–900 mg.).

B. Observations on "primary chill coma"

1. Respiration did not return in any fish subjected to approximately three or more times the LD50 cold exposure, despite the fact that the circulation returned in all fish subjected to approximately twelve times the LD50 exposure or less.

2. The lethality of a cold exposure was increased by decreased oxygen before or during the exposure.

3. The lethality of a cold exposure was decreased by prior anesthesia with 1% urethan, increased oxygen during or after the exposure, or with two sequential cold exposures by exposing the fish to the less extreme temperature first.

4. The lethality of a cold exposure was unaltered by increased oxygen before the exposure, decreased oxygen (of a duration which was not lethal of itself) after the exposure, increased tonicity of the chilling water, or equilibrating the chilling water with 5% CO_2.

C. Observations on "secondary chill coma"

1. The lethality of a cold exposure was unaltered by increased oxygen during the exposure, or by the use of isotonic sodium chloride as the chilling medium.

D. The following conclusions may be drawn from the observations made:

1. Death due to "primary chill coma" in the guppy may be due to anoxic damage to a cold-depressed respiratory center.

2. The lethal effect of "primary chill coma" is related to the profoundness of respiratory depression and by the degree of depletion of the fish's oxygen reserve.

3. Osmoregulative failure does not seem to be a cause of death in "secondary chill coma" in the guppy.

4. Oxygen lack is not a lethal determinant in "secondary chill coma" in the guppy.

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FURROWING IN FLATTENED SEA URCHIN EGGS

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Fifty years ago Yatsu (1908, 1910, 1912) operated with hand-held instruments on cleaving eggs of the nemertine, Cerebratulus and the ctenophore, Beroë. He was able to remove large polar segments from cleaving eggs without interfering with the process. He found that once started in division, the cells demonstrated the greatest pertinacity to divide in spite of operations of the grossest sort. In the years since, many workers have used microdissection techniques on dividing cells: Chambers (1924, 1938) for many exploratory dissections; Dan (1943) to test his astral theory of cytokinesis; Mitchison (1953) in connection with the surface expansion theory; and Hiramoto (1957) to measure the thickness of the cortex during the division cycle. We have used the method to test whether or not "isolated" pieces of furrow cortex undergo contraction. We used the swollen flattened egg as our experimental material because when flattened the mitotic figure is clear, and because cuts can be placed with precision. We have concluded from observations reported here that the position of the furrow is fixed only after elongation has begun, that its position is not necessarily related to the equatorial plane, and that the direction of constriction may be completely altered during the division.

It is not yet possible to adopt any theory of the mechanism of cell division without the gravest reservations. The recent review by Swann and Mitchison (1958) lists eight theories, each one designed to explain a limited set of observations; no over-all theory has emerged, indeed two quite opposite schemes have been proposed to account for the surface changes first observed by Dan, Yanagita and Sugiyama in 1937. According to one of these theories the non-furrow surface expands actively during division (Mitchison, 1952; Swann, 1952); the other theory holds that the furrow cortex contracts actively (Marsland and Landau, 1954). No experiment has yet been reported which will test these two alternatives in a critical way; however, in a previous paper (Scott, 1946), we presented evidence for the continued contraction of the long gelated stalks which occur when eggs cleave in hypotonic sea water. In this paper we present further evidence for the contraction hypothesis.

Material and Methods

Extreme flattening of the sea urchin egg suppresses cytoplasmic division but the division of flattened eggs is facilitated when they are swollen in 60% sea

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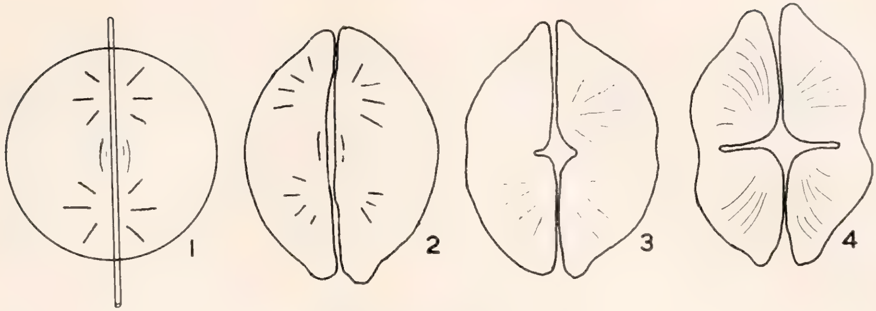
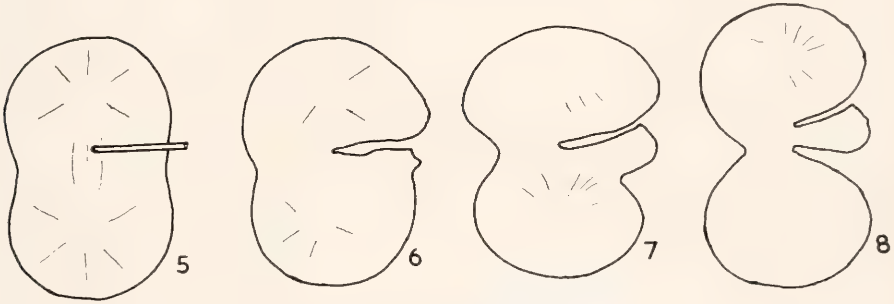
[1] This investigation was carried out during tenure of a National Science Foundation Fellowship. We are indebted to the Stazione Zoologica, and especially to its Director, for facilities furnished and for the general spirit of helpfulness.



OPERATION I



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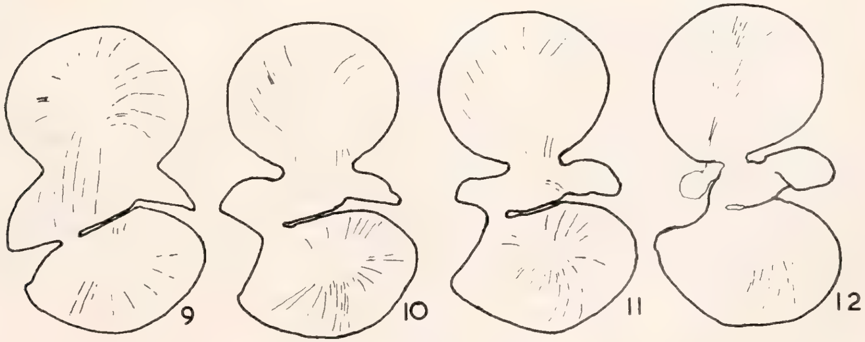


OPERATION II

FIGURES 1–12. Operation I, Figures 1–4, hemisection of the entire egg followed by division of each half egg. Operation II, Figures 5–8 and 9–12, near abscission of large polar segments with completion of furrowing.

water. This seems to be due to a more fluid endoplasm which offers less resistance to the constricting furrow.

The eggs of *Paracentrotus lividus* at Naples are approximately 88 micra in diameter. In 60% sea water they swell to a nearly constant diameter of 100 micra in ten minutes and so increase their volume by 47%. If the swollen eggs are flattened to a diameter of 150 micra their thickness is reduced to approximately 30 micra.

The general technique used was as follows: Eggs were obtained from excised ovaries and were washed twice by settling and decantation. Since there is great variability in the ripeness of different females, tests were made to find a female whose eggs produced high fertilization membranes in at least 95% of the eggs sampled. If control samples of any egg batch failed to cleave with at least 95% normal first cleavage, the egg batch was discarded. The fertilization membranes were removed at three minutes by shaking. At 65 to 70 minutes the eggs were removed to 60% sea water and prepared for microdissection in a hanging drop on a very clean, previously flamed, slide. The degree of flattening was regulated by withdrawing the appropriate amount of the dilute sea water with a fine pipette. Most of the operations were slit punctures made with the flat side of a microneedle. Observations were made on the flattened eggs still mounted in the moist chamber. The manipulator was made by De Fonbrun and the needles were drawn with a De Fonbrun microforge.

RESULTS

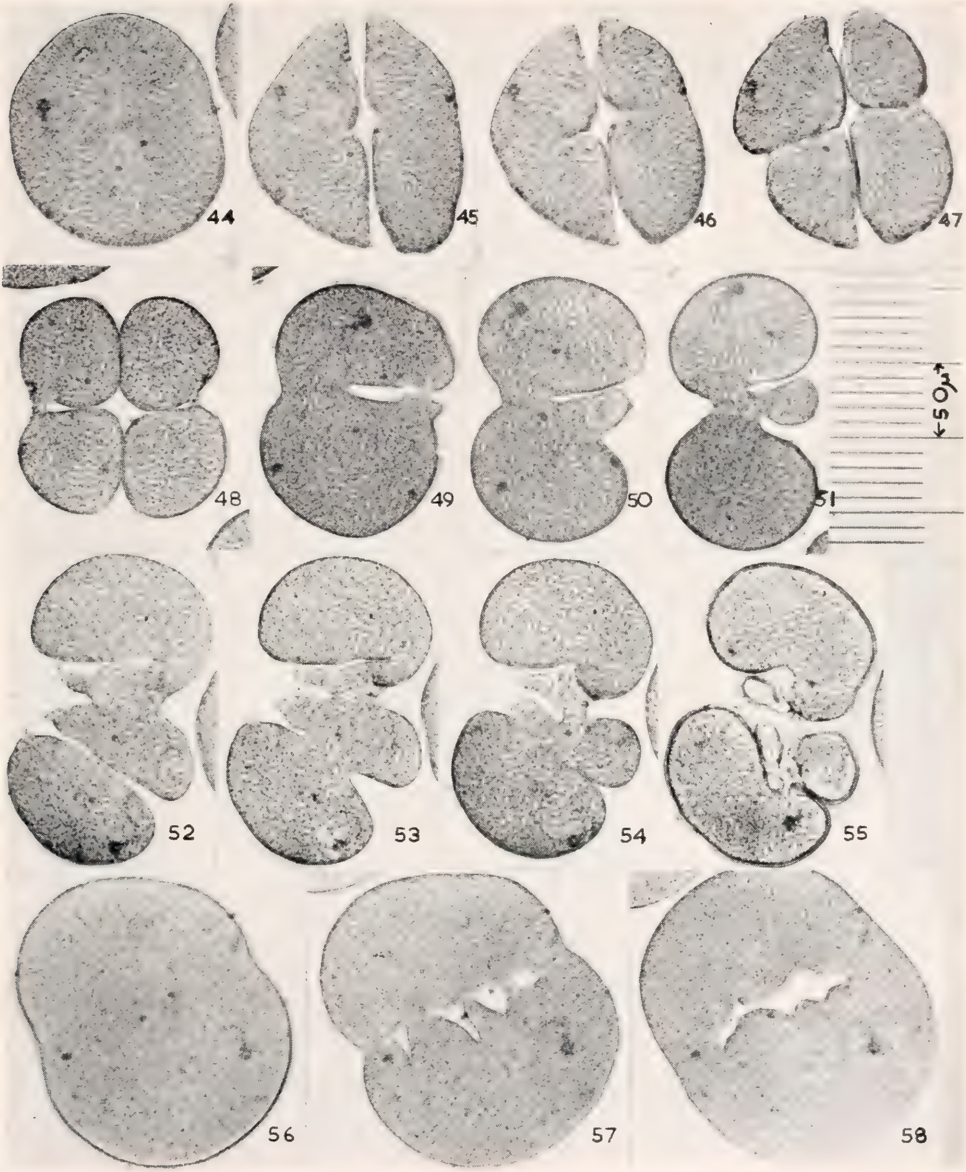
Ten different experiments were performed on the flattened egg. The operations reported involve only the period from mid-diaster, that is, about ten minutes before the first cleavage begins, through the furrowing period. The ten operations are designated I through X and are individually described in the following paragraphs and figured in the accompanying line drawings, Figures 1–43 and photographs, Figures 44–80.

Operation I, hemisection of the entire egg, Figures 1–4 and 44–48:

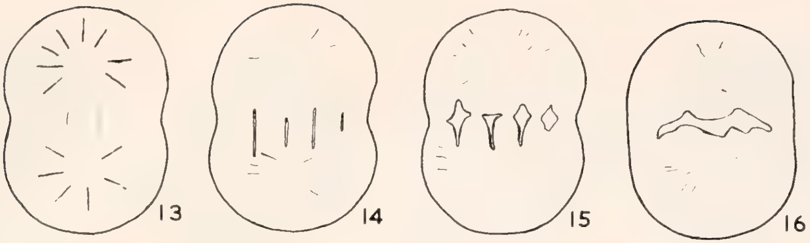
It is surprising to find that when eggs are bisected with a cut which divides the amphiaster neatly through its long axis, both fragments cleave. Each fragment retains visible parts of the half asters and the half spindle. Sometimes the furrow cuts in symmetrically towards the new median longitudinal axis of the half egg or alternatively it cuts out from the plane of hemisection. Yatsu reported that large segments cut from the "side" of the dividing egg completed the furrowing but this observation of cleaving hemisected eggs has a certain elegance when performed on a flattened egg, in that the flatness allows a precise bisection of egg and amphiaster.

Operation II, abscission or near abscission of polar segments, Figures 5–8, 9–12, and 49–55:

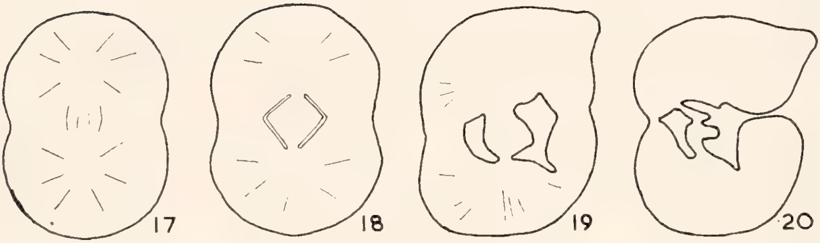
It is possible to cut away one or both poles at the cylinder stage, removing as much as two-thirds of the egg substance, and still get successful furrowing. Neither the position of the furrow nor its course is altered by the operation. This experi-



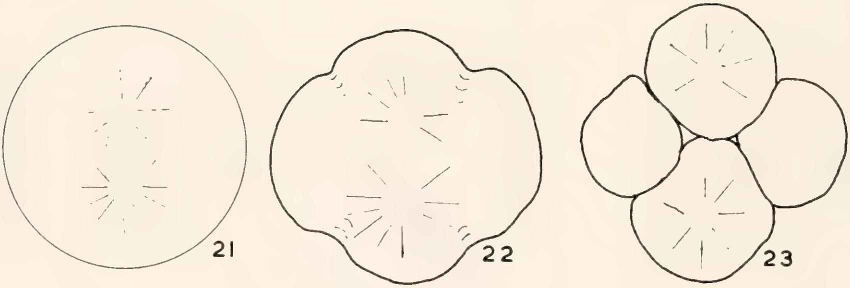
FIGURES 44–58. Photographs illustrating Operations I–III. Operation I, Figures 44–48, hemisection of entire egg with continued furrowing. Operation II, Figures 49–51, continued furrowing after near abscission of one pole. Operation II, Figures 52–55, continued furrowing after near abscission of both poles. Operation III, Figures 56–58, rapid opening of slit perforations (see text). Figure 57 is a few seconds after Figure 56; Figure 58 is 5 minutes later.



OPERATION III



OPERATION IV



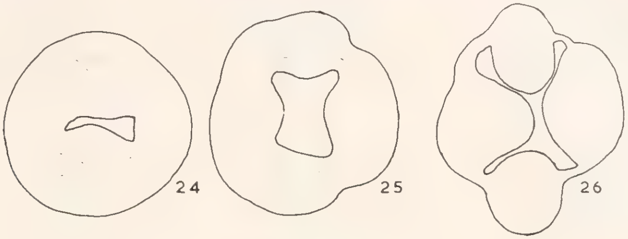
OPERATION V

FIGURES 13–23. Operation III, Figures 13–16, longitudinal slits in the furrow showing the immediate opening-out of the slits and the completion of three internal furrows. Operation IV, Figures 17–20, near isolation of a furrow segment, Figure 18, followed by shrinkage of the segment, Figures 19 and 20. Operation V, Figures 21–23, cleavage of a flattened diastral egg to four cells; two cells with, and two without, aster and nucleus.

ment separates the expanding polar surface from the rest of the egg, during a considerable part of the time that it would be expanding in the unoperated egg, without interfering with cleavage.

Operation III, longitudinal slits in the furrow, Figures 13–16 and 56–58:

During this operation, the egg is flattened in the cylinder stage and one or more slit perforations are made across the furrow with the long axis of the slit parallel



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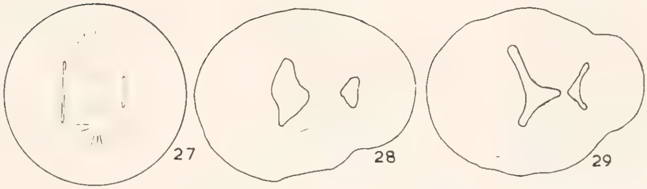
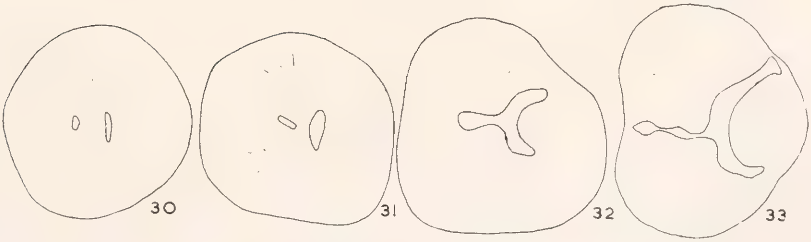
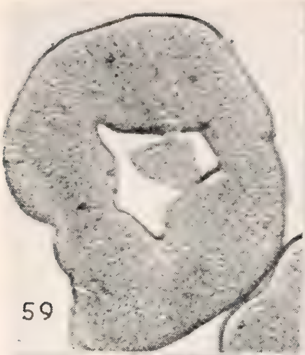


OPERATION VII CASE I



OPERATION VII CASE II

FIGURES 24–33. Operation VI, Figures 24–26, transection of the spindle; development of four furrows and cleavage to four cells, two cells without asters and without nucleus. Operation VII, Case I, Figures 27–29, slit perforations lateral to the spindle; five furrows produce four cells, two without aster or nucleus. Operation VII, Case II, Figures 30–33, slit perforations lateral to the spindle; three cells are formed, one without aster or nucleus.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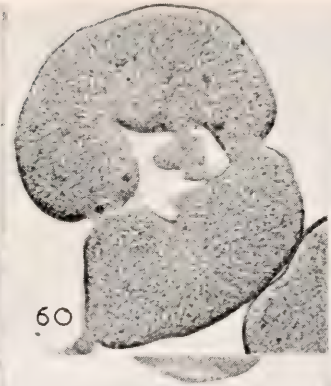
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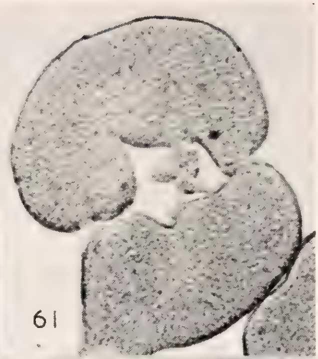
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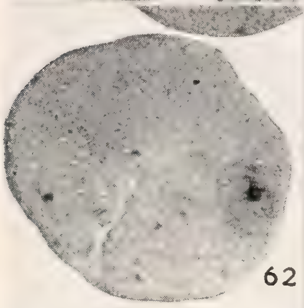
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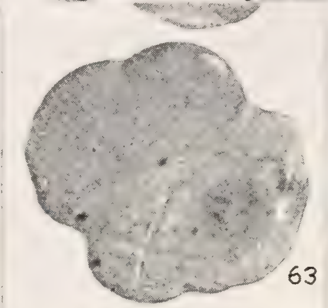
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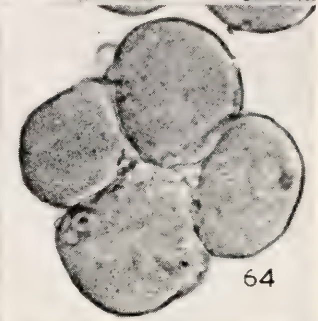
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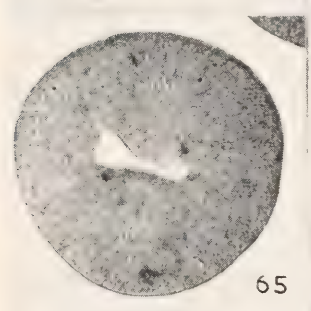
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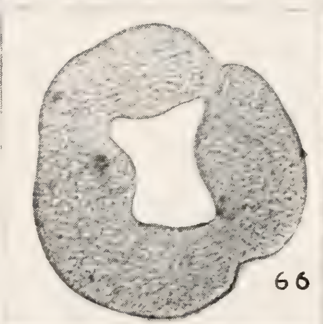
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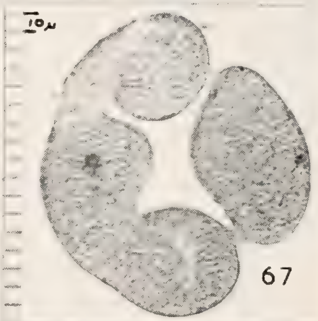
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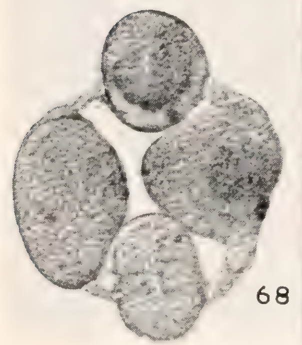
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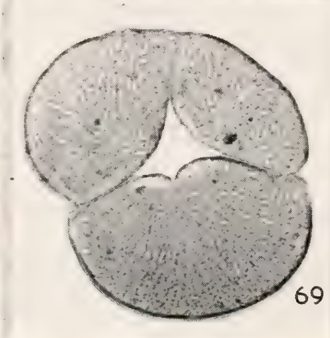
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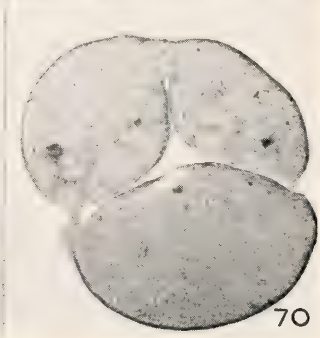
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to the long axis of the egg. The slits immediately open in a way that gives evidence that the furrow ring is a region of tension in flattened eggs. Within seconds the slits open and multiple furrows produced gradually cut through the narrowing stalks. This operation makes cleavage possible in eggs that would otherwise be stalled at the cylinder stage; it is apparently easier for an egg to furrow through several small stalks than through one big one.

Operation IV, near isolation of a furrow segment, Figures 17–20 and 59–61:

In this operation four perforating cuts are made as shown in Figure 18, so that a central plug of egg substance, the surface of which is virtually all furrow cortex, is isolated from the rest of the egg. Only slender stalks connect the plug with the sub-furrow region. During the next few minutes the whole semi-isolated plug shrinks in volume; meanwhile its contained endoplasm flows through the attachments into the main part of the cell. We consider this to result from an active contraction of the entire surface of the plug; it appears to be incompatible with the membrane expansion theory.

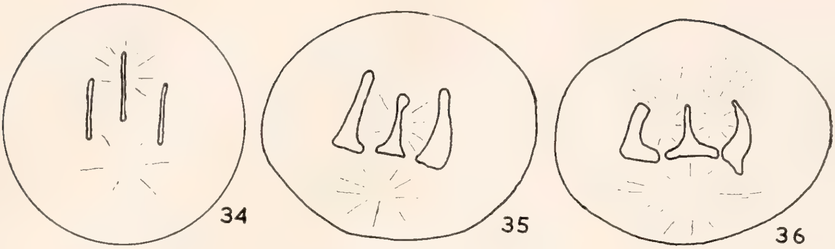
Operation V, flattening without microdissection, Figures 21–23 and 62–64:

When eggs in the middle diaster are swollen, flattened but otherwise unoperated, they may cleave directly to four cells, two of which contain asters and nuclei and two that do not. The expected single equatorial furrow does not develop; instead, four areas of furrowing move in from the periphery following the tips of the astral rays. In two cases the four-celled embryo was followed through a second cleavage during which the nucleated cells divided again while the anucleate ones failed to divide; the latter did, however, each develop a large monaster during the "division phase," which is the more interesting since they were patently devoid of any part of the amphiaster during the first division.

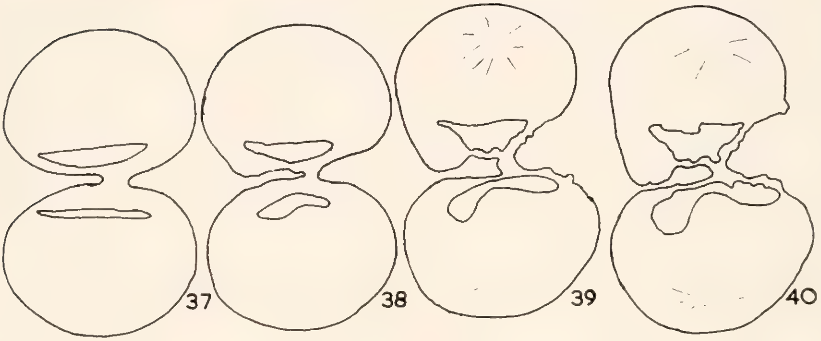
Operation VI, equatorial transection of the spindle I, Figures 24–26 and 65–68:

When one makes a slit-perforation across the equator of the spindle of a mid-diaster stage the perforation soon opens up to form a large hole. The egg then divides directly to four cells, two with nuclei and two without. This is not an occasional result but occurs quite regularly following the transection operation. In all cases with central perforations the furrowing tends to be centrifugal; the furrow cuts out from the hole.

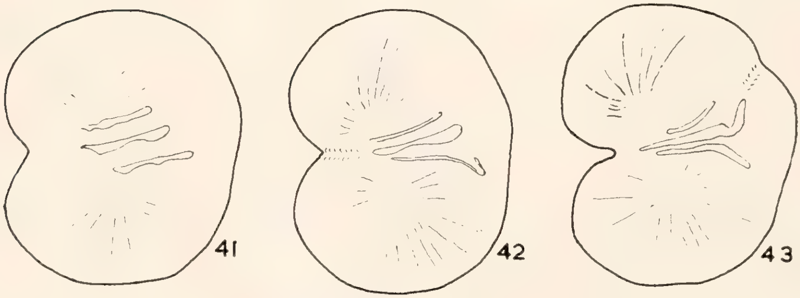
FIGURES 59–70. Photographs illustrating changes after Operations IV, V, VI, and VIII. Operation IV, 59–61, isolation of an island of furrow substance at the cylinder stage showing shrinkage of the furrow island. Operation V, Figures 62–64, flattening without dissection of the furrow with subsequent division of the diastral cell to four cells; two with aster and nucleus and two without. Operation VI, Figures 65–68, transection of spindle of a diastral cell followed by cleavage to four cells; two with an aster and a nucleus and two without. Operation VIII, Figures 69 and 70, bisection of one aster with continued furrowing in the furrow plane but no furrowing between the halves of the divided aster.



OPERATION VIII



OPERATION IX



OPERATION X

FIGURES 34–43. Operation VIII–X, Figures 34–36, bisection of an aster; each half aster forms a spherical aster. Operation IX, Figures 37–40, isolation of walls of completed furrow by means of slit perforations. The tubes of cortex thus formed subsequently shrink in diameter. Operation X, Figures 41–43, isolation of the early furrow cortex by means of slit perforations across the spindle axis. The cortical tubes formed shrink in diameter. Figure 41, immediately after the operation; Figure 43 five minutes later.

Operation VII, slits lateral to the spindle, Case I, Figures 27–29 and 71–73:

Four daughter cells are formed when paired perforations are made on opposite sides of the amphiaster. First there is a furrowing across the spindle equator; then four non-spindle furrows work out centrifugally from the ends of the perforations. In this case as in Operation VI two of the four daughter cells are anucleate.

Operation VII, slits lateral to the spindle, Case II, Figures 30–33:

In some cases three furrows form instead of four, producing two cells with nuclei and one without.

Operation VIII, subdivision of an aster, Figures 34–36 and 69–70:

One or both asters may be bisected with a perforating cut which, if placed as indicated in these figures, will have its inner end drawn out in the furrowing. The half asters become asters of smaller size when bisected and each one has a center from which rays diverge more or less symmetrically. All slits made in the vicinity of asters conform to the sphericity of the aster; apparently even subdivided asters tend to be spherical structures.

Operation IX, "isolation" of late furrow cortex, Figures 37–40 and 74–77:

It is possible to isolate the walls of the completed furrow from the rest of the egg by making two perforations across the spindle axis close to the furrow; this operation forms the furrow walls into tubes which shrink in diameter in the same way as the cleavage stalk.

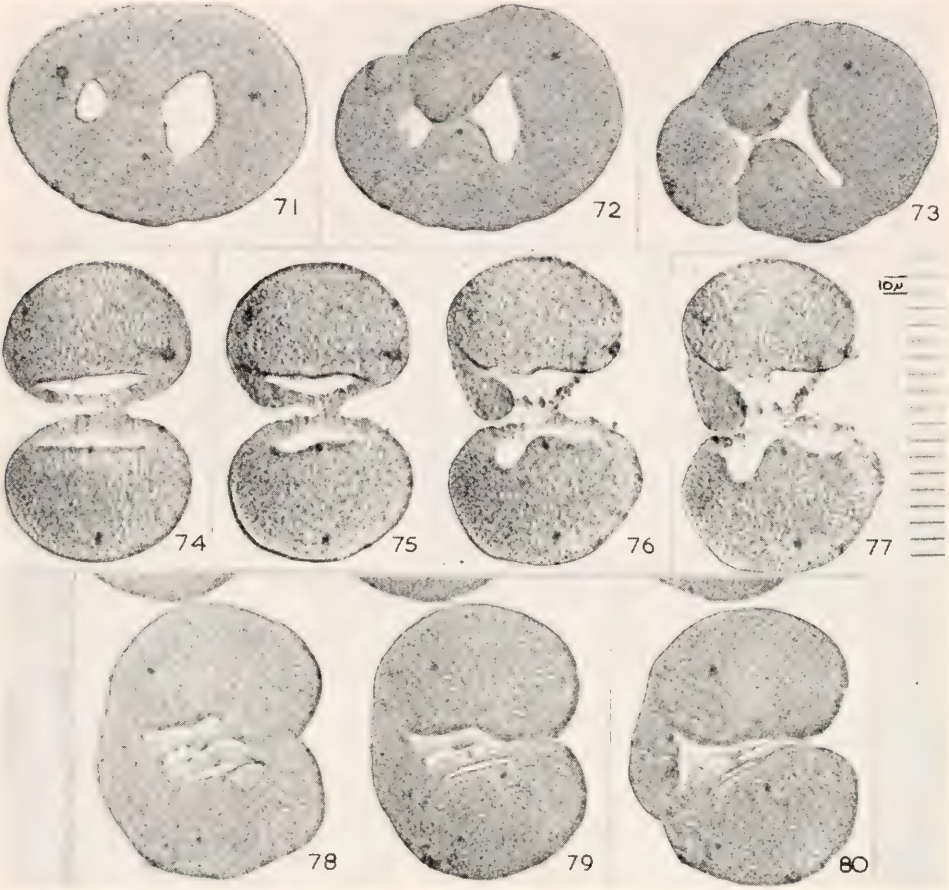
Operation X, "isolation" of the early furrow cortex, Figures 41–43 and 78–80:

In this case the eggs used had just begun to cleave. Two or three slit perforations placed transversely across the mid region isolate tubes of furrow cortex which diminish in diameter like furrow stalks, but their direction of constriction is quite different from the presumptive one.

Discussion

Contraction

We have taken the position that the weight of evidence now favors the theory that cell cleavage occurs by active, cortical contraction. The "proof" will be more compelling if a contractable substance can be extracted from the cell cortex as it can be from muscle. Indeed such proof may be near, for Hoffmann-Berling (1954, 1960) has reported the extraction of a contractile protein from fibroblasts and from sarcoma cells which may be responsible for the furrowing *in vivo* and in his glycerine-water-extracted telophase cell models. One of the strongest indications that the cortex contracts is the shrinkage of the "furrow islands" reported in this study which, although isolated early in furrowing, shrink progressively to become thin strands. In other experiments we produced tubes of furrow cortex



Figures 71–80. Photographs illustrating operations VII, IX, and X. Operation VII, Case I, Figures 71–73, slits lateral to the spindle; five furrows form four cells, two with aster and nucleus and two without. Operation IX, Figures 74–77, isolation of tubes of late furrow cortex followed by shrinkage of the tubes. Operation X, Figures 78–80, isolation of tubes of early furrow cortex followed by shrinkage of the tubes.

by a number of different operations (*cf.* Fig. 18 and Fig. 59), after which only narrow connections joined the tubes to the main mass of the egg, and in all cases the tubes diminished in diameter in the manner of a cleavage stalk. Such phenomena are difficult to explain by the membrane expansion theory (Mitchison, 1952; Swann, 1952) which hypothesizes that the expansion of non-furrow surface causes the shrinkages of the furrow. In our experiments furrow cortex is nearly separated from the non-furrow surface, yet it shrinks very effectively. One could argue that the expansion process reaches into the furrow itself but this view must be discarded, as Swann and Mitchison (1958) have pointed out, because there is no area of expansion in the furrow (Dan and Ono, 1954).

Furrow tension

Our experiment III indicates that a circumferential tension is present around the furrow ring by the time of cell elongation. This is contrary to measurements with the elastimeter (Mitchison and Swann, 1955), for no measurable differences in stiffness were found over the surface of the egg at the cleavage stage. We note here that while the eggs used in our studies were swollen, flattened, and certainly abnormal in many ways, still, they did cleave and almost certainly by the same mechanism as normal eggs. Tension around the equator is, as Mitchison and Swann have pointed out, inconsistent with the expansion theory. In their consideration of the flattened egg they have assumed (Mitchison and Swann, 1955) that the tension produced by flattening disappears before expansion begins. They suggest that this would explain the slower furrowing observed after flattening. In the case of experiment III, however, the tension seems never to fall to "zero," for each slit of a series, made one after the other, opens quickly within seconds, indicating that tension still exists around each smaller furrow. The failure to observe tension differences at cleavage with the elastimeter may perhaps be due to the fact that the endoplasm is a fairly viscous gel which resists being drawn into the instrument. Real differences in the stiffness of the cortex would then be masked. It was found that the general stiffness of the surface increases while the asters are quite small, indicating that the aster is not then affecting the measurement, but the "stiffness" value of thixotropic endoplasm is difficult to assess.

What determines where a furrow will form?

In these experiments we have utilized two stages of division for the most part, the one a mid-diaster stage and the other about ten minutes later, at the beginning of cell elongation. The evidence supports the view that the furrow cortex is unspecified during the earlier period and that a definite band of furrow cortex, with special properties, is determined from the moment of cell elongation. It is not possible to perform operations on elongated eggs which will provoke furrowing in areas outside the presumptive furrow. The furrowing cortex is therefore determined; it can be notched, isolated in islands and variously perforated, but it retains its capacity for involvement in constriction. The position of the furrow is not fixed at mid-diaster since simple spindle transection, for example, produces three or four furrows in regions quite different from the presumptive one. What, then, determines where a furrow will form? It has been almost universally accepted that the position of the furrow is related to the spindle equator; thus polar body formation involves such a relationship, whatever the size of the two cells produced, as does micromere formation, the unequal first cleavage in mollusks and abnormal cases involving multipolar spindles. Now, however, the idea of coincidence of mid-spindle and furrow must be abandoned. In our view furrow formation involves three factors:

1. The presence of an aster (not necessarily two), since furrows always follow an astral boundary.

2. The recognition by the cell of a region of narrowness. Deformed cells (see Figure 67) are curiously "opportunistic" in their ability to exploit a region

of narrowness as a potential furrow path. Perhaps the surface in these isthmian regions is differentially stretched and differentially sensitive to furrow initiation.

3. The presence of some (chemical) initiator which may affect the entire circumference of the potential furrow or which may be limited initially to one region of the circumference acting as a furrow head. It seems necessary to assume that the old concept of "furrow head" (Ziegler, 1898) has some validity since many cases occur in which the furrowing process moves progressively over the surface. Yatsu, for example, demonstrated (1910) that the furrow head would bifurcate after some operations disturbing its presumptive path. We conceive of the furrow head in some of these operated eggs as a sector of contraction involving only a part of the furrow ring, the other sector being passive at first, then progressively involved as contraction spreads around the furrow. We conceive, furthermore, of the whole furrow as being under tension as soon as any part of it contracts. This would explain the deep incursions which occur on one side of some furrows while the other side is barely indented. The problem is to explain how an initiator substance appears at precisely those places where furrows will form, for example, at the ends of the slits in Operation III.

Orientation of contractile units

We have shown that operations on the elongated egg may change the orientation of furrowing in areas where it has already begun. We have elsewhere noted other cases in which new directions of furrowing set in (Scott, 1946), for example, in the late furrowing of dispermic eggs where the plane of constriction around the neck of a one-quarter blastomere is displaced 45° from the original plane. In the course of another study, as yet unpublished, we observed changes in the patterns made by echinochrome granules located in the presumptive furrow cortex of cleaving *Arbacia punctulata* eggs. We noted that the furrow surface shrinks two-dimensionally during the earliest phases of the cleavage, from the sphere to the cylinder stage, after which it shrinks only around the circumference of the furrow. We speculate, therefore, that the contractile elements of the furrow cortex are arranged at random in the plane of the surface but that effective contraction can only occur in directions where resistance to shrinkage is low. According to this interpretation the contractile elements of the band of furrow cortex are arranged at random in the plane of the surface; during the sphere-to-cylinder stage they all contract whatever their orientation, but during the subsequent stages of the furrowing only those elements oriented around the furrow are able to shorten. This hypothesis can obviously be extended to include the unusual "furrow" patterns resulting from micromanipulation, for example Figures 74–77 and 78–80. In these latter examples, cylinders of furrow substance shrink in directions quite different from the presumptive ones.

SUMMARY

1. Eggs swollen in 60% sea water cleave effectively when flattened.

2. Furrowing continues after abscission of one or both poles of the egg.

3. Furrowing is initiated and completed after hemisection of the still spherical egg through the long axis of the amphiaster.

4. Bisection of the spindle just before egg elongation causes a diastral egg to cleave into four blastomeres, two of which lack asters.

5. Islands of furrow cortex "isolated" at early furrowing shrink progressively during division. This is regarded as evidence for active furrow contraction.

6. Tubes of furrow cortex prepared from the furrow walls shrink in directions quite different from the original one.

7. The furrow cortex is not determined until the beginning moment of elongation for cleavage.

8. The position of the furrow is not necessarily related to the plane of the spindle equator.

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SURFACE CHANGES DURING CELL DIVISION

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The general features of surface change during cell division have been known for the sea urchin since the inspired work of Dan and his colleagues (Dan, Yanagita and Sugiyama, 1937). Dan's original observations have been substantially confirmed by Hiramoto's excellent analysis (1958). In the present paper we, too, confirm Dan's original observations, although we cannot support his theoretical interpretation (Dan, 1943). The Japanese workers made use of adherent clay or carbon particles to mark the surface; we have used, instead, the echinochrome granules of the egg cortex as natural markers. The detailed behavior of these intensely red granules is of interest because they are fixed in the gelated cortex and there is no possibility of slippage between the surface and the marker. Photographs of the granule patterns give a faithful record of large areas of the cell surface in a way that a few adherent particles can not do. It should be noted that Dan also used the echinochrome granules to mark the movement of the cortex in cleavage but with another aim in view and at much lower magnifications (Dan, 1954). We describe in this paper the surface behavior of a few eggs with some care. The photographs show a "new view" of furrow and pole during division and give especially clear evidence of the polar surface change. The drawings were prepared in every case by tracing photographs.

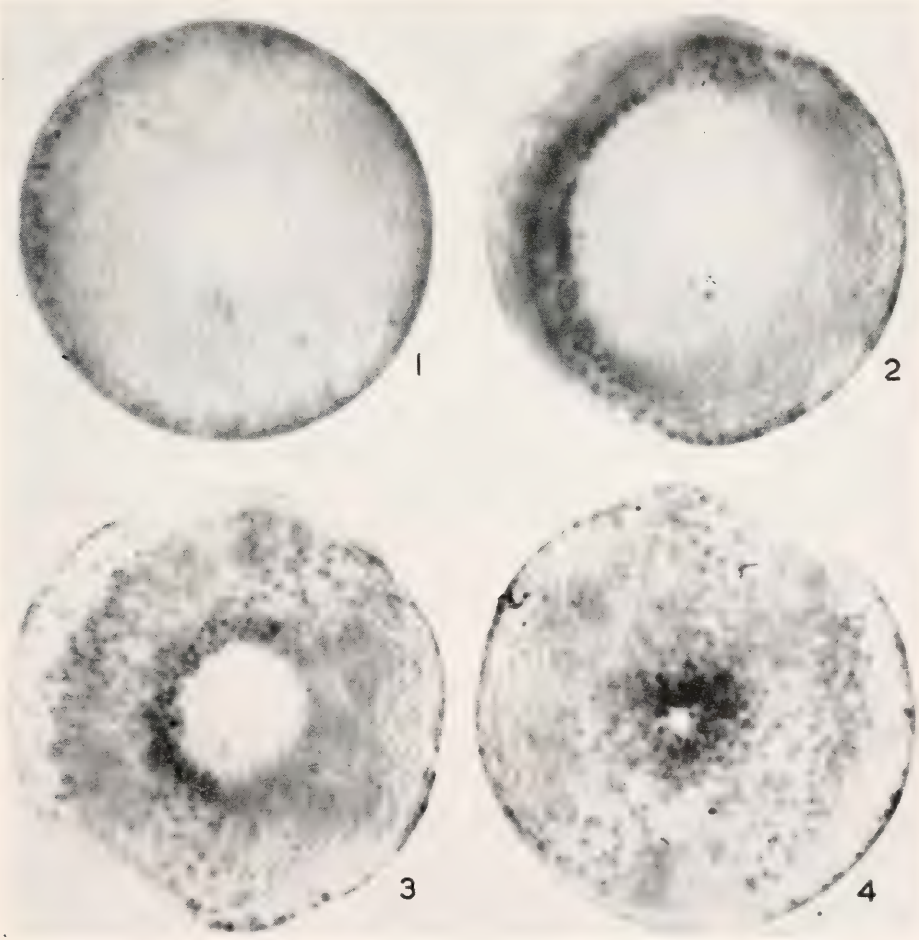
The term "pole" as used here refers to points on the egg surface normally intersected by the spindle axis. The furrow is considered to have the cell-equatorial plane through its middle. Latitudes are parallel to the equator and longitudes run from pole to pole.

MATERIAL AND METHODS

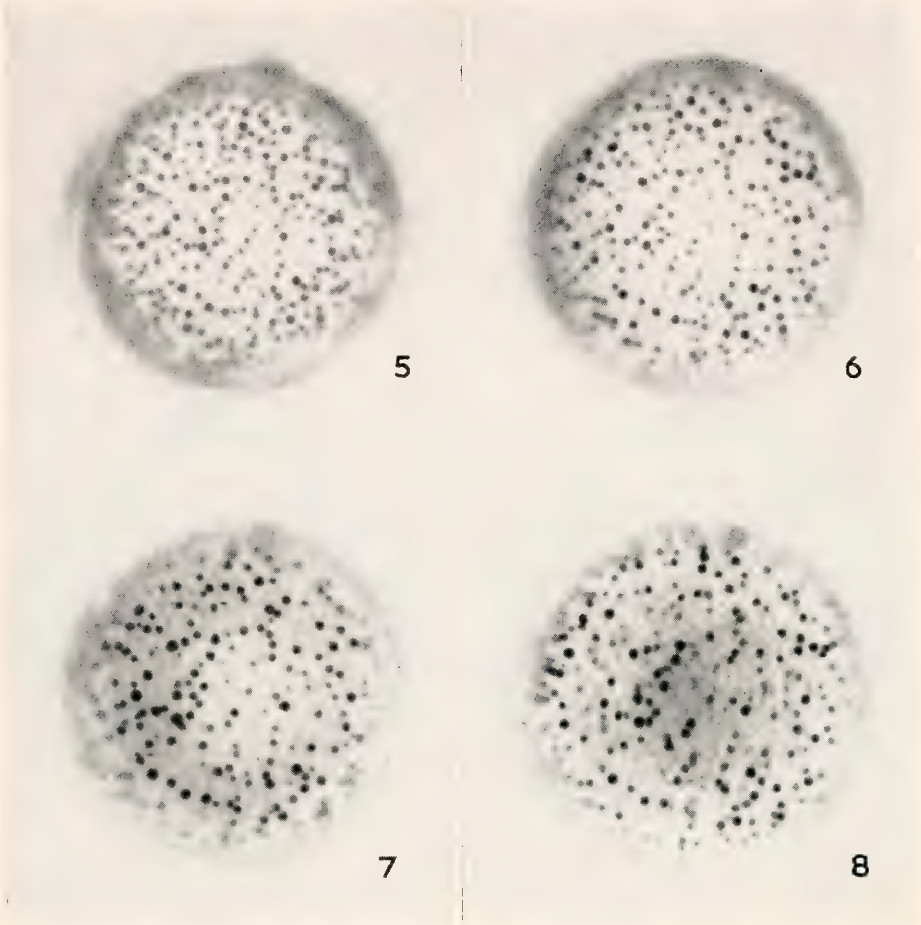
The red echinochrome granules of the *Arbacia punctulata* egg stand out in strong contrast in the green light of a Wratten No. 58 filter. The individual granules can be photographed sharply through substantial thicknesses of cytoplasm, so that both the surface granules and those deep inside the egg can be seen clearly. The eggs of some females have much darker granules than those of others and, moreover, egg batches differ in the average size of the granules and in the average number of granules per egg.

Eggs and sperm were obtained by the electrical method (30 volts A. C.). All of the egg batches used showed at least 95% of the eggs with high fertilization membranes and at least 95% cleavage of the fertilized controls. The fertilization membranes were removed three minutes after insemination by shaking. Just before the cleavage was due, the eggs were transferred to a cold stage held at about 10° C. Cold dry air was blown across the cold stage to prevent condensation; with this apparatus it was possible to take sequential photographs of the same region of the surface showing the granule patterns in sharp focus. Photographs of the polar surface and views of the furrow seen from the polar axis were taken with the microscope in horizontal position. The eggs were mounted between cover and slide and sealed with Vaseline. When the microscope was tilted into the horizontal position, the eggs came to rest on a glass hair. An occasional cleaving egg was found to be oriented with its long axis coincident with the optical axis. With this technique one polar surface and the equatorial (furrow) plane were observed with good resolution. The inner furrow margin was seen to close like an iris diaphragm, as in Figures 1, 2, 3 and 4. The division of quarter blastomeres can be viewed in this aspect on the upright microscope.

Polyspermic eggs show multiple and intersecting furrows during the first cleavage, in which the regions of furrow intersection show characteristics not found in



Figures 1–4. Views of the equatorial plane from the polar axis, showing the iris-like closure of the furrow ring.



FIGURES 5–8. Views of one polar surface of the egg pictured in Figures 1–4. The following pairs of photographs were taken within a few seconds of each other: 1 and 5; 2 and 6; 3 and 7; 4 and 8. Figures 1 and 5, cylinder stage. Figures 4 and 8, at the end of furrowing.

normal, diastral cleavage. Polyspermy was induced by methods detailed previously (Scott, 1946).

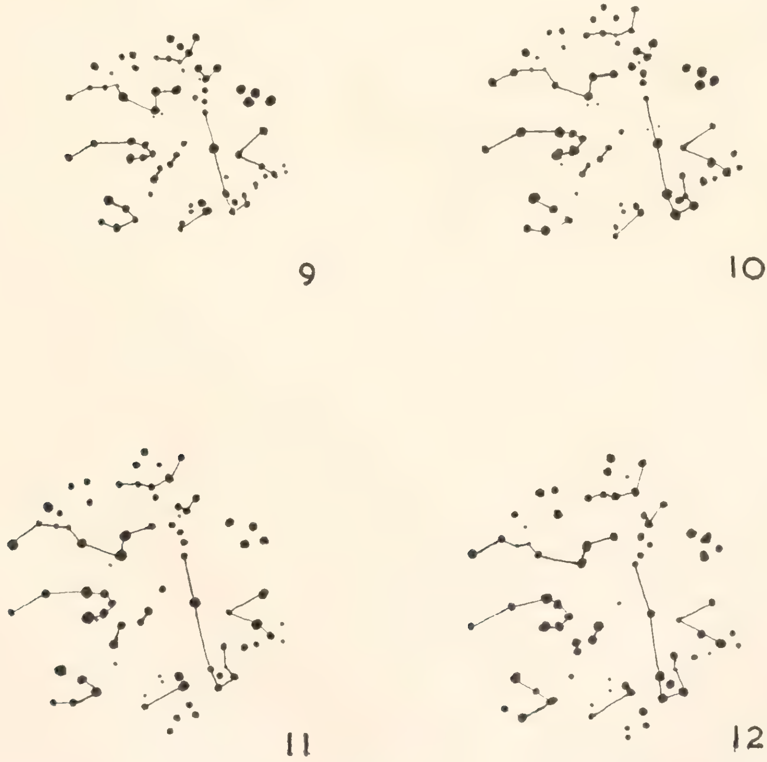
RESULTS

Cortical stiffness indicated by granule patterns

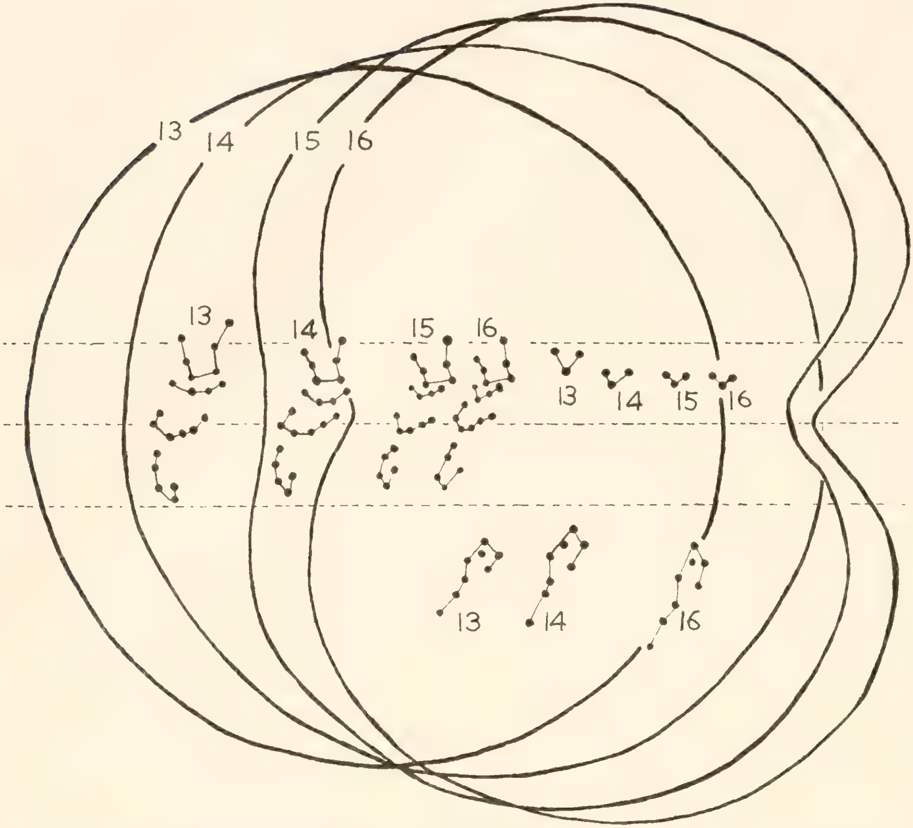
The movements of the echinochrome granules are as follows: (1) In the unfertilized egg they are scattered throughout the cytoplasm; within seven minutes after fertilization at 20° C. most of them have migrated to, and become fixed in, the cortex. (2) The individual granules when observed with oil immersion are seen to undergo a slow random migration within the cortex up until the moment of cell division. (3) During furrowing the cortex is gelated enough to hold the granules in relatively fixed positions so that patterns persist until the end of furrowing when the cortical gel softens and the patterns blur as the granules resume their slow random movement.

Polar surface

The polar surface stretches during furrowing as is illustrated in Figures 5, 6, 7 and 8. The polar surface expands during the first part of the furrowing but remains stretched without further expansion from the time the inner furrow diameter is about 10 micra to the end of the process. The amount of increase in area of the polar zone shown in the photographs is in excess of 65% and the degree of radial expansion is essentially the same for any sector of the polar surface. Constellations of granules have been connected by lines in drawings made from the photographs above (Figs. 9, 10, 11 and 12). In some eggs the expansion continues until the end of furrowing, while in others the expansion is finished by the time the inner furrow diameter reaches ten micra.



FIGURES 9–12. Drawings made from photographs 5–8; the constellations of granules have been connected by lines to make the granule patterns more evident. Polar expansion occurs during the interval between 9–11. There is little change in the polar surface area in late furrowing (compare Figures 11 and 12).



FIGURES 13–16. Egg outlines and corresponding surface granule patterns during four stages of cleavage from the sphere stage to early indenture. Patterns shrink two-dimensionally in Figures 13–14 but only in directions around the furrow in Figures 15 and 16.

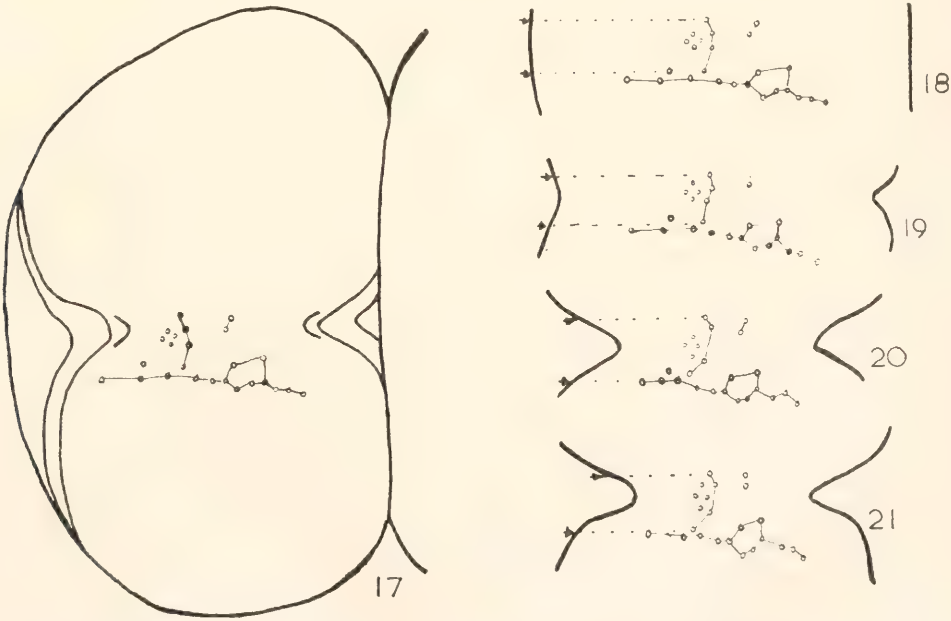
Furrow surface

During the earliest phase of cytokinesis the presumptive furrow region flattens around the equator. This is the first phase of surface shrinkage in the potential furrow region. Equatorial shrinkage starts even before cell elongation is apparent. Figure 13 represents an egg just before cleavage is to start. It shows a wide granule pattern on one region of the future furrow surface, while Figure 14 shows the same region a little later when the egg is still nearly spherical. Comparison of Figures 13 and 14 shows that equatorial shrinkage is two-dimensional at this stage in the plane of the surface. The furrow area shrinks along lines of longitude as well as around latitudes. At least one-tenth of the egg surface is surely involved in this shrinkage which represents a band about 15 micra wide around the equator. A longitudinal expansion of the furrow begins at the cylinder stage (Figs. 15 and 22) but its extent can only be appreciated when granule positions are projected to the furrow margin.

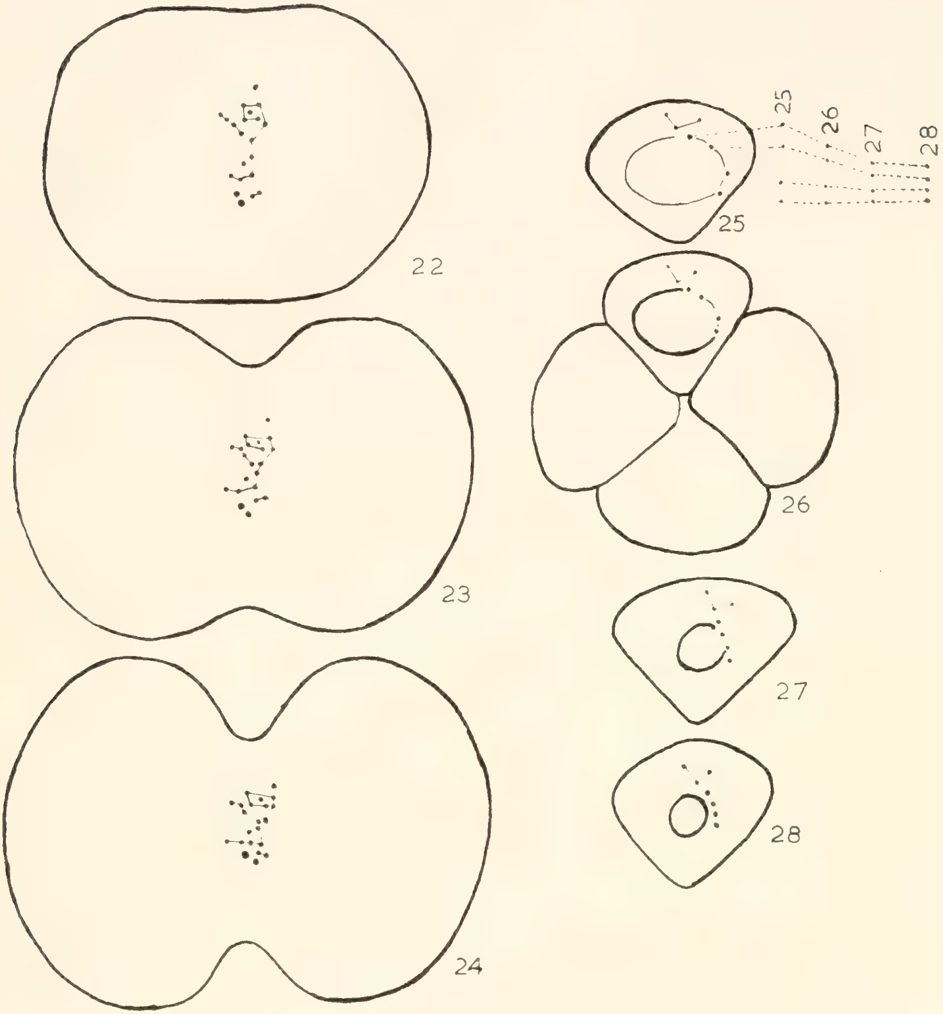
Views of the exact center of the furrow show the plastic deformation of the surface that occurs in that region. Granule patterns fixed there are deformed by the narrowing equator and by extension in the long axis shown in Figures 17–21. A circumferential shrinkage of the furrow continues throughout cytokinesis, shown also in Figures 17–21, where a row of granules undergoes a steady proportional reduction in length as the cell divides. When viewed from the pole, the furrow closes down like an iris diaphragm, and it is clear that a circumferential shrinkage is occurring throughout this process because granules located on the inner furrow margin are crowded together in a close row as the "iris" boundary moves in. The shrinking circle of granules is clearly seen in Figures 25–28, which illustrate the inner furrow margin in one blastomere during the third cleavage. The successive positions of four granules are diagrammed in four stages of the division. We observe here a shrinkage of this arc to be approximately proportional between each pair of granules. Thus the shrinking substance, whether actively contracting or passively compressed, is shrinking uniformly around the furrow. The granules noted in this case fell behind in the final stages of the furrowing and the furrow peak pushed on beyond them. Shrinkage ends earlier in paraequatorial latitudes than in the equator proper.

Intersecting furrows in polyspermic eggs

Triastral and tetrastral eggs involve intersecting furrows, and at the points of intersection there is a special area of surface whose fate is different from the rest.

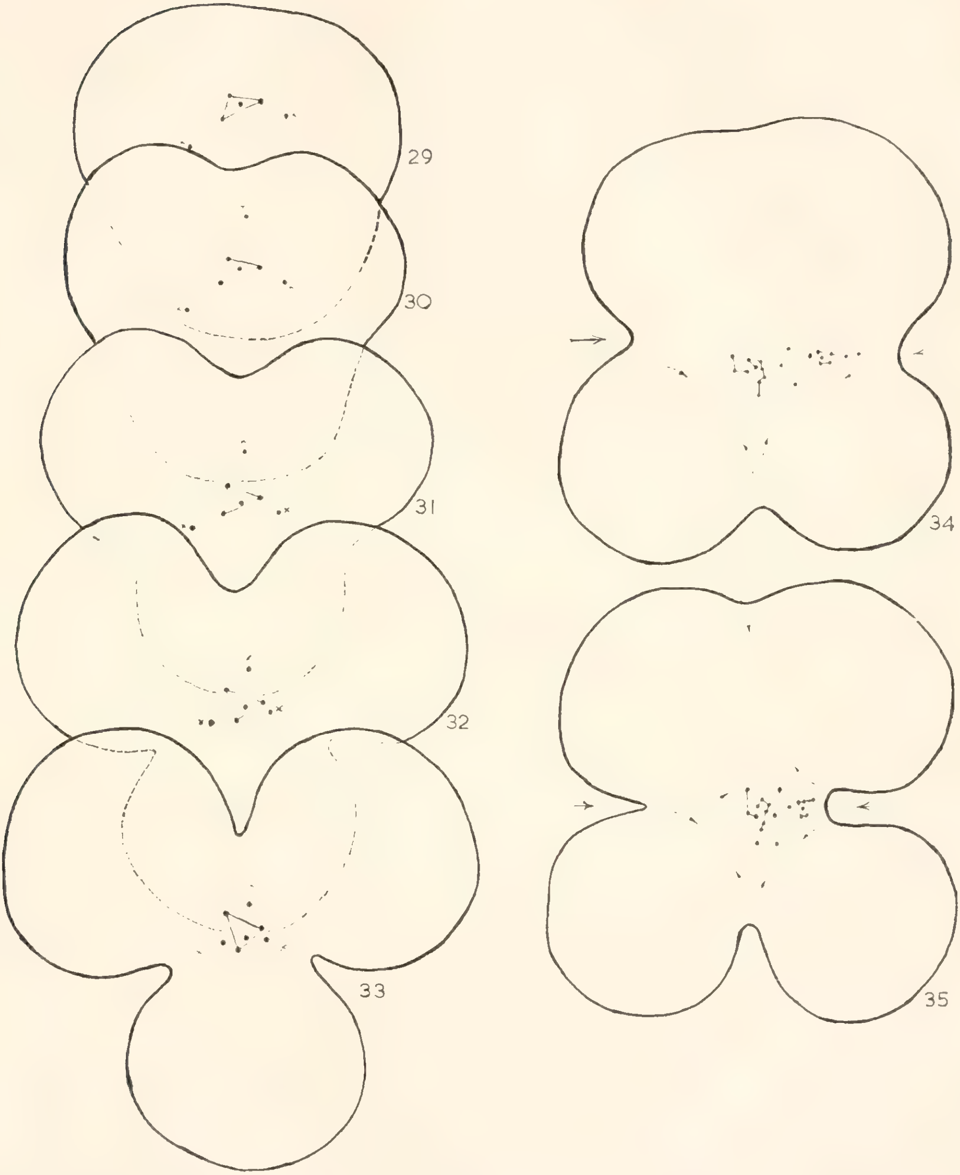


FIGURES 17–21. Granule patterns in mid-furrow and sub-furrow. FIGURE 17. One egg at second cleavage, showing the location of granule patterns in the furrow area. FIGURES 18–21. Details of stages of division in the egg shown in Figure 17, showing circumequatorial shrinkage of patterns on the sub-furrow surface and plastic deformation of patterns at the furrow peak.



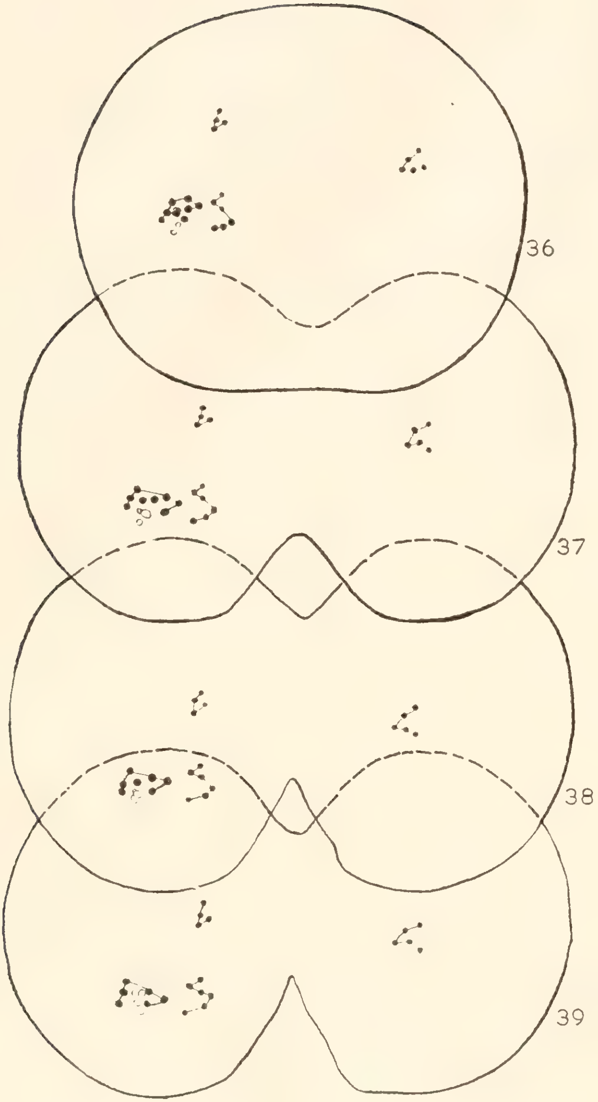
Figures 22–28. Granule patterns in the furrow region. Figures 22–24. Deformation of granule patterns in the deepening mid-furrow. Figures 25–28. View from polar axis, showing iris-like closure of the inner furrow margin of a one-quarter blastomere involving four stages of shrinkage of a pattern of four granules. The relative linear distance between the granules at each stage is illustrated in the numbered diagrams connected by dotted lines.

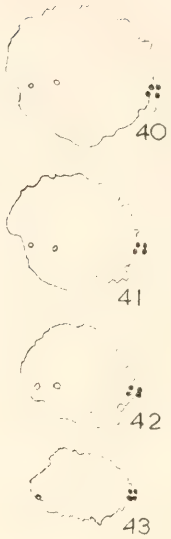
Such eggs flatten like biscuits as they go off round (Scott, 1946). The flattened surfaces represent regions where the shrinkage is greatest in the initial stage, and vectors of shrinkage occur in more than one direction. They are first marked by concentrations of granules at the animal and vegetal poles. During the formation and the deepening of the multiple furrows, the central island shrinks only in directions tangential to the neck of incipient blastomeres. A triastral cleavage (Figs. 29–33) shows that shrinkage occurs around the blastomere neck so that granules

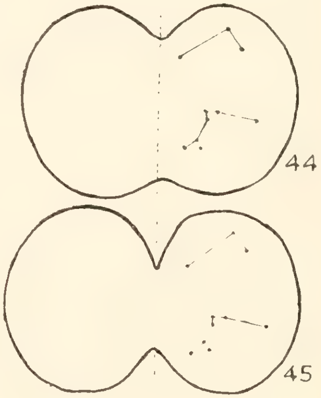


FIGURES 29–35. Intersecting furrows in polyspermic eggs. FIGURES 29–33. Triastral cleavage. A circumferential shrinkage in the furrows is shown by the movement of granules xxx; the area marked by the triangle does not shrink.

FIGURES 34–35. Tetrastral cleavage. Granule patterns indicate circumferential shrinkage in the dominant furrow and stretching in a direction at right angles.







FIGURES 36–45. Granule patterns on the lateral surface and in the endoplasm. FIGURES 36–39. Lateral surface of the egg, showing the relative immobility of granule patterns. Ishizaka's stationary ring. FIGURES 40–43. Furrow plane and inner furrow margin of an egg in first cleavage; four granules imbedded in the cortex ride on the margin while endoplasmic granules remain motionless. FIGURES 44–45. Endoplasmic granule patterns in a mid-optical section move away from the furrow plane with little distortion.

x-x-x converge but there is a central island, marked by the triangle, which does not shrink, although it might be expected to on the basis of the surface expansion theory (Mitchison, 1952; Swann, 1952). Two stages of a tetrastral cleavage are shown in Figures 34 and 35. In this case one furrow, at the arrow, is in advance of the other and the area of intersection behaves as an integral part of the more advanced furrow. There is marked shrinkage of the surface between the arrows while the "island" surface is, in fact, elongating in the plane of the delayed furrow.

Lateral surface

Ishizaka (1958) has disclosed that two ring areas about the cell can be regarded as stationary if their position during furrowing is related to the center of mass (equator region) and if the spindle axis is fixed. He called them the stationary rings. The position of the rings is determined on two-dimensional outline figures and the term "stationary" refers essentially to the unchanging distance between the ring plane and the equatorial plane on a two-dimensional figure. Now, actually, and Ishizaka realized this, the ring boundaries move further from the equator in a three-dimensional figure when over-the-surface distances are considered. It does not astonish us that the longitudinal expansion of the furrow and of the pole compensate at the latitude of the rings and that they do indeed remain essentially stationary (Figs. 36–39), but while there is little translation of this lateral surface, it does undergo a steady stretching in a longitudinal direction during the whole of the furrowing (see the same Figures 36–39). It results, then, since the lateral surface is relatively fixed, that the subdividing endoplasm within undergoes massive translocation towards one or the other pole.

Endoplasm

A few echinochrome granules remain in the endoplasm and it can be seen that those located in the furrow plane, but outside the spindle, lie quietly in place until the inner furrow margin reaches them (Figs. 40–43). Granules located in a mid-longitudinal plane, away from the furrow, are moved, with some distortion of pattern, further away from the equatorial plane (Figs. 44 and 45). Evidently most of the endoplasm is a deformable plastic mass rather than fluid.

Discussion: Which Theory of Cytokinesis?

Spindle-thrust. In many ways our observations represent a thorough-going confirmation of those made by Dan and his associates. We have substantiated the initial shrinkage of the presumptive furrow. We concur as regards polar, subpolar and subfurrow stretch during furrowing. Yet while we confirm the data, we must reject Dan's spindle-thrust theory. If the 1908 polar abscission experiments of Yatsu on the egg of *Cerebratulus* are not sufficiently conclusive, surely the demonstration that furrowing continues after removal of the amphiaster (Hiramoto, 1956), has made any astral-thrust theory untenable.

Cortical growth. Observations on the newt egg give compelling evidence that a new unpigmented cortex forms in the endoplasm below and ahead of the forming furrow (Selman and Waddington, 1955) and the authors believe that contraction of the new cortex is a factor in the cell division. It is unlikely that a similar process occurs in the sea urchin egg for the following reasons: (1) all of the equatorial endoplasm is translocated away from the equator as the furrow cuts in; (2) the furrow advances while endoplasm pours through the furrow constriction from one blastomere to the other (in hypotonic calcium-free sea water) (Scott, 1946); (3) cleavage continues smoothly during continual agitation of the furrow endoplasm with a microneedle (Mitchison, 1953).

Surface expansion or furrow contraction. No experiment has yet been designed that will give a clear answer as to whether the active force for cytoplasmic division rests in furrow contraction or in expansion of the non-furrow surface. Our reasons for rejecting the expanding surface theory (Mitchison, 1952; Swann, 1952) are the following: (1) There is now substantial evidence that the furrow cortex has different properties than the non-furrow surface, despite the inability of Mitchison and Swann (1955) to measure a physical difference with their ingenious elastimeter; Beams and King (1937) separated a cleavage-active material from the *Ascaris* egg by ultracentrifugation; Marsland (1950) has demonstrated critical pressure-temperature characteristics of the furrow gel. The furrow cortex has been shown to respond in special ways to hypotonic treatment (Scott, 1946); to dinitrophenol and azide (Kuno-Kojima, 1957). The latter worker has, moreover, isolated fragments of furrow cortex with acid and a detergent. (2) We observed (Scott, 1946) that furrowing may occur in hypotonic sea water with one blastomere increasing in volume while the other is shrinking. This is clearly incompatible with expansion theory. (3) We agree with Marsland and Landau (1954) that surface expansion alone could not provide a vector of force which would direct the surface inward beyond the cylinder stage. (4) The islands noted on trispermic eggs do not undergo continued shrinkage in the way that they would be expected to if active polar expansion were compressing the island from several directions.

In the face of the equivocation still remaining, the contraction theory seems simplest; as Marsland and Landau have noted (1954), it requires no second factor, such as a mechanism for spreading the expansion into the furrow, to explain the "dipping in" after the cylinder stage. What we consider to be strong evidence for the contraction theory was presented in our 1946 paper. It was noted that long stalks develop between blastomeres in hypotonic solutions which, although tenuous, are rigid enough to push the blastomeres 18 micra apart while they continually diminish in diameter.

We can visualize the active furrow surface as a band of specialized cortex about 15 micra wide on the uncleaved egg. We recognize it as different from the rest of the cortex by virtue of the fact that it shrinks, at least in two dimensions, in the initial stage of furrowing. This band of special cortex shrinks most in the center of the furrow and less so on each side, merging into a region which never shrinks at all in the longitudinal direction. Furrow surface shrinkage, which is two-dimensional until the cylinder stage, is circumferential only, around planes parallel to the equator, from the cylinder stage on. The question arises as to why the furrow cortex, previously capable of two-dimensional contraction, is now limited to circumferential contraction around the equator. One might assume that the potentiality for two-dimensional contraction still exists and could assert itself at any time that resistance could be overcome, but the now cylindrical form and the semi-solid structure allow only the circumfurrow vector of contraction to operate while the longitudinal vector is overcome and the furrow surface expands in the longitudinal direction. In this way we are able to explain the oriented contraction without assuming a pre-orientation of contractable micelles around the equator.

We consider that our observations are in substantial agreement with the contraction hypothesis as formulated by Marsland and Landau (1954). In one detail, however, we can not support their theoretical views. According to their hypothesis, the cortical gel at the furrow peak undergoes solation, with the result that subfurow cortex moves into position at the furrow peak. The authors put it as follows (Marsland and Landau, 1954, p. 532): "Thus more and more cortical gel, possessing an unexpended fund of contractile energy is brought into an operative position as the furrow deepens. Therefore, in the final stages of furrowing, to complete the cleavage it is only necessary to assume that the region of active contraction shifts from the trough of the furrow to the side-walls, first to the region immediately adjacent to the trough and later, to a more peripheral site, somewhat removed from the trough (Stages 4 and 5). In this way, the gel at the very bottom of the trough, having performed its contractile function, could undergo solation, clearing the way for the approach and final fusion of the cell membrane, which severs the stalk between the daughter cells." We have observed, however, that the echinochrome granules are scattered throughout the thickness of the cortex; we have seen no evidence of a non-granular under-layer, which, if present, might undergo solation. Furthermore, as far as we can tell, no furrow granules are liberated to the endoplasm during division—either at the furrow peak or elsewhere. What one observes in fact, is a steady, circumferential contraction of the furrow cortex from the beginning to the end of the process; the cortex changes its shape exceedingly but maintains its integrity throughout. It is possible that new material possessing contractile capacity is added *among* the granules of the furrow, and that the material whose contractile power has been exhausted is expelled, but the basic fabric of the furrow cortex is not destroyed and in any case no granules escape to the endoplasm.

SUMMARY

1. A study of cell surface changes during cell division has been made, using echinochrome granules as natural markers rather than adherent bits of clay or dye.

2. The polar surface of the sea urchin egg stretches symmetrically in all radii from the pole during the first two-thirds of furrowing, and either remains at maximum stretch or shrinks somewhat during the latter third.

3. The furrow surface shrinks in two dimensions in the plane of the surface during earliest furrowing to the cylinder stage; it shrinks latitudinally in a degree which is proportional to the distance from the equator and it expands longitudinally from the cylinder stage on; the cortex undergoes plastic deformation at the peak of the furrow; there is no liberation of cortical echinochrome granules to the endoplasm at any stage of division.

4. All of the cortex is relatively rigid throughout division. The first signs of softening occur at the poles during late furrowing.

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MECHANISMS OF REMOVAL OF INJECTED MICROORGANISMS FROM THE AMERICAN OYSTER, CRASSOSTREA VIRGINICA (GMELIN)[1, 2, 3]

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Extensive studies on the amoebocytes[4] of oysters have elucidated their importance in digestion, but these cells have not been extensively studied as regards their role in defense. It is clear that in this filter-feeding mollusc, amoebocytes move freely through the gut epithelium and engulf partially digested particulate food (Yonge, 1926; Takatsuki, 1934). Intracellular digestion follows and the products of digestion are presumably made available to all the cells of the body. In other Mollusca, complex digestive organs are present and intracellular digestion plays a minor role (Yonge, 1937). In such animals amoeboid cells associated with certain tissues and organs act as specialized defensive agents (Metchnikoff, 1901; Cuénot, 1914).

Stauber (1950) demonstrated that carbon particles (India ink) injected intracardially in the American oyster, *Crassostrea virginica,* are taken up by phagocytes and removed from the tissues as these cells migrate through epithelia to the exterior. However, intracardially injected red blood cells are eliminated both by migration of phagocytes and by intracellular digestion (Tripp, 1958). These studies suggest that two separate defense mechanisms are involved in the oyster, and a study of the relative importance of these processes in relation to injected microorganisms serves as the basis for this report.

MATERIALS AND METHODS

1. *Maintenance of oysters* (*C. virginica*)

The oysters used in these experiments were obtained from either Delaware Bay or the Navesink River at Red Bank, New Jersey. They were thoroughly scrubbed with a wire brush to remove encrusting organisms and mud, and then were maintained in groups of eight or fewer in three-gallon Pyrex battery jars containing one gallon of sea water. This water was of the same salinity as that from which the oysters were originally obtained and was changed twice daily. All aquaria were constantly aerated and were maintained at 17 ± 1° C.

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[1] Part of a thesis submitted to the graduate faculty of Rutgers, the State University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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[3] This investigation was supported by a research grant (E-781-C) from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health.

[4] The terms amoebocyte, phagocyte and leukocyte are used interchangeably. The cells referred to are found free in the blood and are both amoeboid and phagocytic.

In preparation for intracardial injection a small hole was made (using a coarse wood rasp or a ¼″ electric drill) in the left valve of the shell, directly over the heart. Once the nacreous layer had been penetrated, the opening was carefully enlarged with forceps until the entire heart was visible. A scalpel incision through the mantle and pericardial membrane then exposed the heart. These wounds began to heal within a few days and many oysters formed new shell before they were sacrificed.

Preliminary experiments demonstrated that the adductor muscle was also suitable for injection. The muscle bundles are bathed by extensive blood channels which drain directly into systemic vessels so that material injected into the muscle very quickly reaches the systemic circulation. In preparation for intramuscular injections both valves of the shell dorsal to the adductor muscle were filed so as to expose the muscle, caution being taken to avoid injury to the subjacent tissue.

Prior to injection each oyster was appraised as to its general physiologic condition in terms of strength and regularity of heart beat, frequency of shell closure, and ejection of fecal strings. Animals which did not appear normal were discarded.

2. *Maintenance of microorganisms*

All microorganisms selected for injection could be recognized by colonial characteristics in agar pour plates and, thus, could be distinguished from the endogenous bacterial flora of oysters.

A strain of *Bacillus cereus* var. *mycoides* (*B. mycoides*) was obtained from the Institute of Microbiology, Rutgers, the State University. Cultures of *Staphylococcus aureus, Escherichia coli, Pseudomonas fluorescens* and *Saccharomyces cerevisiae* were obtained from the Department of Bacteriology, Rutgers, the State University. *Flavobacterium invisibile* was isolated from oyster heart blood. Microorganisms were subcultured routinely on nutrient agar slants, but for purposes of preparing inocula, organisms were grown as outlined in Table I.

All cultures in liquid media were harvested by centrifugation and washed three times with 15 ml. of sterile 0.85% saline. Spores were harvested from agar plates

Table I

Media, incubation times and temperatures for various microorganisms used in injecting oysters

Organism	Medium	Incubation	
		Temp. (°C.)	Time (Days)
Bacillus cereus var. *mycoides*		25	5
spores	Nutrient agar (dist.)*	then 5	7
vegetative cells	Nutrient broth (dist.)	25	3
Staphylococcus aureus	Nutrient broth (dist.)	37	1
Escherichia coli	Nutrient broth (dist.)	25	1
Pseudomonas fluorescens	Nutrient broth (dist.)	25	1
Flavobacterium invisibile	Nutrient broth (sea)	25	1
Saccharomyces cerevisiae	Sabouraud broth (dist.)	25	2

* Difco Bacto-nutrient agar, broth or Sabouraud broth rehydrated with distilled or sea water.

and were similarly washed. Final suspensions of organisms were prepared in autoclaved sea water of the same salinity as that in which the oysters were maintained. Prior to injection, the concentration of microorganisms in each inoculum was estimated by dilution plate counts.

3. *Injection procedures*

Spores and vegetative cells of *B. mycoides*, and vegetative cells of *Staph. aureus*, *F. invisibile* and *S. cerevisiae* were injected intracardially. Vegetative cells of *E. coli* and *P. fluorescens* were injected into the adductor muscle.

All injections were done with a 1.0-ml. tuberculin syringe fitted with a 27-gauge ($\frac{3}{4}''$) or a 30-gauge ($1\frac{1}{2}''$) needle. Each oyster was placed in sea water and observed with a binocular dissecting microscope while the needle was inserted directly into the ventricle or adductor muscle; 0.2 ml. of inoculum was injected over a period of several seconds. Oysters received 10^5–10^9 microorganisms, depending on the species of organism and the nature of the experiment. Withdrawal of the needle allowed the wound to close and little of the inoculum was lost. Animals from which excessive leakage occurred were discarded. Each inoculated oyster was passed through six changes of fresh sea water, during which the area over the injection site was flushed gently with a pipette to remove extraneous microorganisms. The oysters were then returned to aquaria and maintained as described above.

4. *Sampling procedures*

Two types of experiments were performed in order to determine the fate of injected microorganisms. In one group of experiments oysters which had received intracardial injections of *B. mycoides* or *S. cerevisiae* were relaxed, fixed, and prepared sections were examined microscopically. In the second group of experiments tissues from experimental oysters were cultured (at intervals) to recover microorganisms.

a. Sections of inoculated oysters

Preliminary experiments showed that tissue shrinkage due to fixation and dehydration could be minimized by relaxing intact oysters in 0.4 *M* magnesium sulfate at 4° C. for several hours prior to fixation of the whole oyster in Bouin's solution overnight. Ten oysters were injected intracardially with a suspension of *B. mycoides* and one animal was sacrified at each of the following intervals after injection: 1 hour, and 1, 2, 4, 5, 8, 9, 12, 16 and 20 days. Similarly six oysters were injected intracardially with a suspension of *S. cerevisiae* and one animal was sacrificed at each of the following intervals thereafter: 1 hour, and 1, 2, 3, 4 and 12 days. Two or three control oysters were injected with equal volumes of sterile sea water and sacrificed at suitable intervals in each experiment. Examination of control animals disclosed no histological abnormalities or microorganisms.

Following initial fixation the soft parts were removed from the shell, divided transversely into six approximately equal parts and placed in fresh Bouin's solution for 1–4 hours. These blocks of tissue were then washed, dehydrated, cleared, imbedded in paraffin, sectioned at 10 μ and stained with Gram's tissue stain. Two sections were taken from each block, one representing the anterior edge of the tissue and one from the center of the tissue. Thus, for each animal 12 transverse sections were obtained at regular intervals along the anteroposterior axis.

Certain vessels, sinuses and tissues were regularly examined for microorganisms under high dry and oil immersion objectives. These included: heart, anterior and posterior aortae, large arteries of the visceral mass, subepithelial blood sinuses of the gut, sinuses between digestive diverticula, dorsal and ventral circumpallial arteries, proximal mantle vessels, medial and lateral gill axis sinuses, vertical gill vessels, palp arteries and the blood spaces of the adductor muscle and kidney. In addition, the following epithelial areas were examined for microorganisms: walls of the alimentary canal at all levels, walls of twenty of the digestive diverticula, inner and outer aspects of the palps, outer aspect of the mantle, nephridial tubules, gonaducts, epicardium and pericardium.

b. Cultivation of microorganisms from tissues

Groups of 20–25 oysters received injections of microorganisms suspended in sterile sea water, while 6–8 controls were injected with sterile sea water only. Estimates of the numbers of microorganisms in heart blood, mantle, visceral mass and in aliquots of homogenized whole oysters were made at intervals of one hour, one day, two days, and irregularly thereafter for 6–50 days.

At each interval three experimental and one control oysters were tested for microorganisms according to the following scheme. Each heart yielded 0.1–0.5 ml. of blood which was added to 4.5 ml. of sterile distilled water. Portions of tissue, approximately 0.1 ml. in volume, were removed from the mantle and visceral mass and ground separately in Ten Broeck tissue grinders containing 5.0 ml. of sterile distilled water. The remaining soft parts were homogenized in a Waring Blendor containing 20 ml. of sterile distilled water, and the actual volume of the soft parts was determined by displacement. One-tenth ml. of the material from each tissue or homogenate sampled was placed in each of three Petri dishes, melted agar added and the plates incubated at the optimum temperature for the species

TABLE II

Media, incubation times and temperatures for bacteriologic tests of oyster tissues

Organism	Medium	Incubation	
		Temp. (°C.)	Time (Hours)
Bacillus cereus var. *mycoides*			
spores	Nutrient agar (dist.)*	30	24
vegetative cells	Nutrient agar (dist.)	30	24
Staphylococcus aureus	Nutrient agar (dist.)	37	24
Escherichia coli	Nutrient agar (dist.)	37	24
Pseudomonas fluorescens	Nutrient agar (sea)	25	48–72
Flavobacterium invisibile	Nutrient agar (sea)	30	18
Saccharomyces cerevisiae	Sabouraud agar (dist.)	30	18–24

* Difco Bacto-nutrient agar or Sabouraud agar rehydrated with distilled or sea water.

of microorganism being studied. Individual colonies were counted, averaged and the number of viable microorganisms per ml. of sample was calculated. Using these data, and estimates of the volumes of mantle and visceral mass (respectively 30% and 40% of the soft parts) derived from previous experiments, it was possible to estimate the total number of viable microorganisms in these organs. Results are expressed as the mean values from three oysters. In general, values obtained were in close agreement and only rarely were extreme variations noted. Since there was no suitable method of estimating the total volume of oyster blood, the number of microorganisms recovered from blood samples is expressed per ml. of heart blood sampled.

RESULTS

1. Observations based on tissue sections following injection of yeast cells

Masses of yeast cells seemed to occlude main arteries immediately after intracardial injection. Phagocytosis began immediately and by 24 hours phagocytes contained approximately 95% of yeast cells visible in sampled areas of tissue sections. Tissue sections from the oyster fixed at 24 hours showed that most leuko-

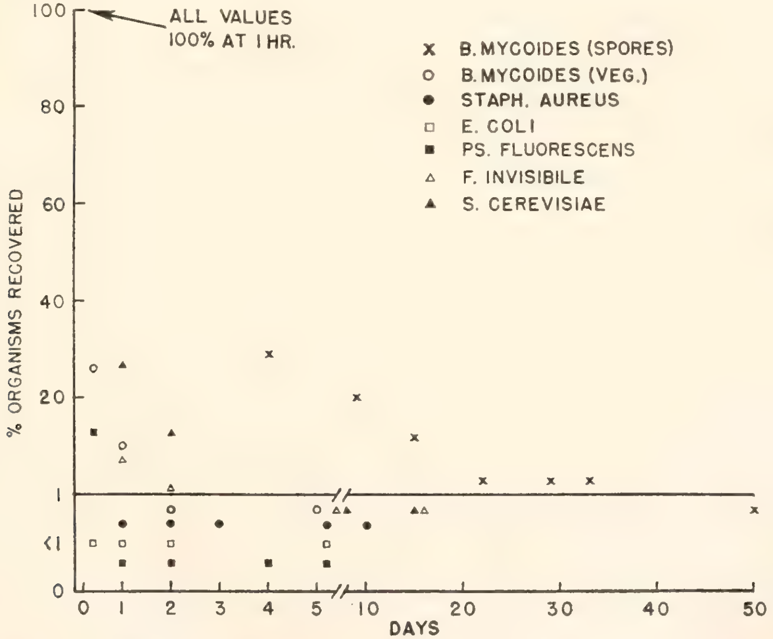

FIGURE 1. Survival of injected microorganisms per ml. sample of oyster heart blood taken at intervals up to 50 days. Base line samples at one hour represent 100% recovery, and subsequent results are expressed proportionately. Each point represents the mean number of microorganisms from three oysters sampled. Points in the area labeled "< 1" are not quantitative measurements, but indicate that small numbers of bacteria were detected for several days following injection. The last point shown for any one microbial species indicates the termination of a sampling series.

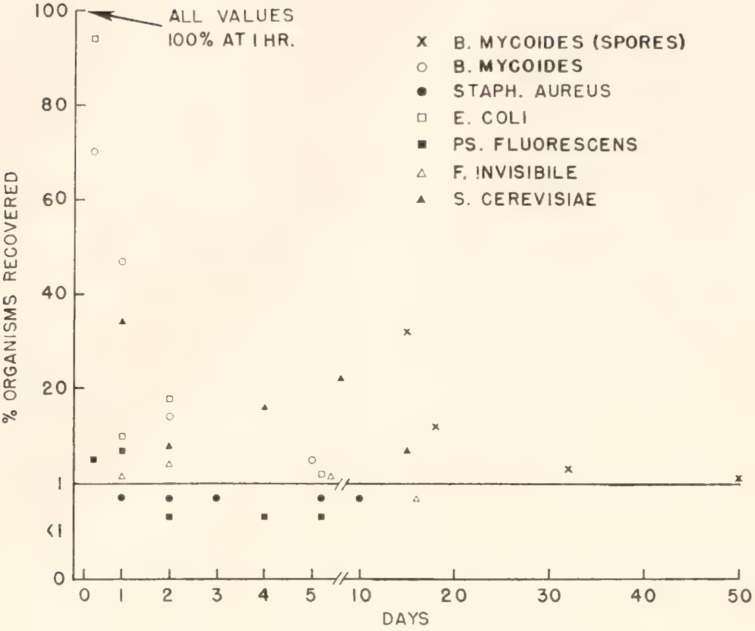

FIGURE 2. Survival of injected microorganisms in samples of oyster mantle tissue at intervals up to 50 days. Base line samples at one hour represent 100% recovery, and subsequent results are expressed proportionately. Each point represents the mean number of microorganisms from three oysters sampled. Points in the area labeled "< 1" are not quantitative measurements, but indicate that small numbers of bacteria were detected for several days following injection. The last point shown for any one microbial species indicates the termination of a sampling series.

cytes contained 2–4 yeast cells, some 8–10 yeast cells, and a few as many as 15–20 yeast cells. It was also evident that appreciable numbers of yeast-laden leukocytes had begun to migrate from blood vessels into adjacent tissues.

During the second day phagocytosis and migration progressed with the result that although individual yeast cells were still present in smaller vessels, large masses of yeast cells were no longer present in the main vessels. Phagocytes containing yeast cells were first seen in the epithelium lining the gut and the digestive diverticula at 48 hours. By the third and fourth day a relatively massive investiture of yeast-bearing phagocytes was evident in the epithelium of the gut, digestive diverticula, palp and mantle, but rarely were such leukocytes found in the epithelium of the gills, kidney, pericardium or gonaducts. Few yeast cells remained in any of these tissues at 12 days post-injection and it appeared that the majority of yeast cells had been eliminated by the migration of yeast-bearing leukocytes to the exterior.

Substantial variations observed in the morphological and tinctorial properties of a small proportion of yeast cells were thought to represent the effects of intracellular digestion. The normally oval or circular outline of some yeast cells became irregular as early as the fourth day post-injection, and instead of appearing a uniformly deep blue these yeast cells were mottled or very pale blue. Ultimately, only faintly staining globules, thought to represent the remains of yeast cells, were seen intracellularly. Morphologically-altered yeast cells were rarely seen outside of phagocytes.

2. *Observations based on tissue sections following injection of B. mycoides*

Suspensions of *B. mycoides* injected intracardially were composed mainly of short chains of vegetative cells which could be detected in Gram-stained tissue sections up to the ninth day. Examination of heart blood smears and tissue sections taken one hour post-injection clearly showed that large numbers of bacteria had already been phagocytized. Tissue sections from the oysters fixed at one hour and later disclosed that phagocytized bacteria were transported within a few hours from major blood vessels into adjacent tissues, while non-phagocytized bacteria remained in the blood vessels. Within 2–5 days post-injection most of the phagocytized bacteria were removed from the oyster by phagocytes migrating through the gut epithelium, or through the inner or outer surfaces of the mantle.

Phagocytized microorganisms which failed to be eliminated via an epithelial

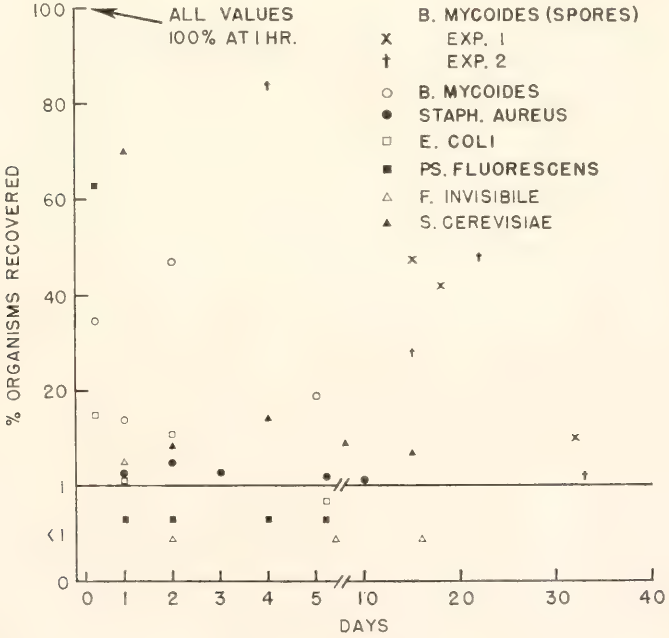

FIGURE 3. Survival of injected microorganisms in oyster visceral mass tissue at intervals up to 33 days. Base line samples at one hour represent 100% recovery, and subsequent results are expressed proportionately. Each point represents the mean number of microorganisms from three oysters sampled. Points in the area labeled "< 1" are not quantitative measurements, but indicate that small numbers of certain bacteria were detected for several days following injection. The last point shown for any one microbial species indicates the termination of a sampling series.

surface underwent vacuolization and loss of Gram-positive character, suggesting that they were dead or dying. Similar changes occurred among extracellular organisms during the first 48 hours after injection. Such degenerative changes were observed only very rarely in control (sea water) suspensions of bacteria maintained at room temperature for similar periods of time.

Except for local accumulations of leukocytes, the significance of which was difficult to evaluate, no distinct pathology was observed in sections of injected oysters.

3. Results of culturing living microorganisms from tissues

Recovery of viable microorganisms from samples of blood, organs and homogenized whole oysters resulted in two distinct groupings of data, one for bacterial spores and the other for vegetative bacteria or yeast (Figs. 1–4). While only a relatively small proportion of injected vegetative cells could be recovered and only within 24–48 hours after injection, bacteria injected as spores were recovered in relatively larger proportions over a period of several weeks. Although the inocu-

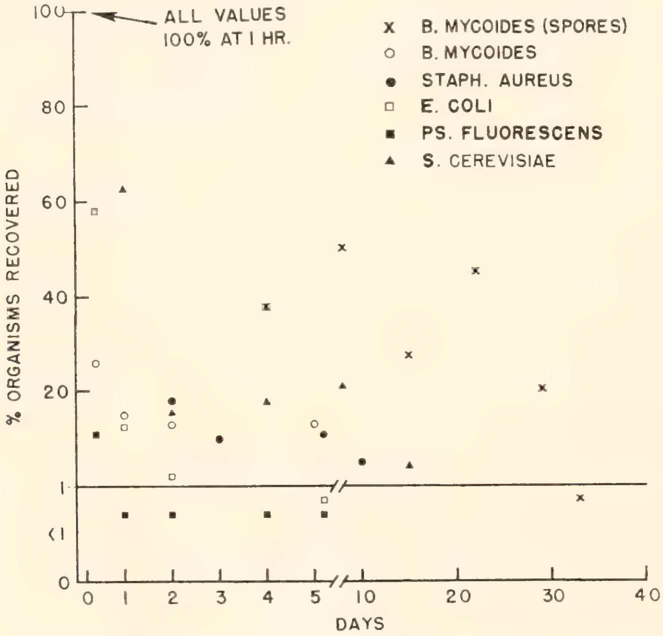

FIGURE 4. Survival of injected microorganisms in whole oysters homogenized at intervals up to 33 days. Base line samples at one hour are assumed to represent 100% recovery, and subsequent results are expressed proportionately. Each point represents the mean number of microorganisms of three oysters sampled. Points in the area labeled "< 1" are not quantitative measurements, but indicate that small numbers of certain bacteria were detected for several days following injection. The last point shown for any one microbial species indicates the termination of a sampling series. (Data for *F. invisibile* are omitted because of contamination of samples.)

lated microorganisms were not completely removed, there was no evidence of bacterial multiplication.

COMMENT

It is evident that injection of any of several foreign particles into the oyster elicits a basic cellular response. Studies reported here indicate that injected yeast cells or bacterial spores were disposed of by the oyster in the same manner as carbon particles (Stauber, 1950). In all of these cases particles became clumped in efferent vessels and large numbers of leukocytes accumulated around them; most particles were engulfed by leukocytes, removed from the circulation and subsequently transported through epithelial layers to the exterior. These processes are initiated immediately after injection, continue until most foreign particles have been removed and may be supplemented by intracellular and/or extracellular destruction. On the other hand, the oyster's defense against injected vegetative bacteria seems largely to be dependent upon intraphagocytic digestion. It is possible that oysters maintained under more favorable conditions (*i.e.,* in larger volumes of sea water) would eliminate injected microorganisms even more rapidly. It would be of interest to determine the fate of an oyster pathogen under such circumstances.

TABLE III

Method of removal	Present study			Tripp '58	Stauber '50
	Bact. spores	Yeast	Veg. bact.	R.B.C.	Carbon
Phagocytosis and migration	++++	++++	+	++	++++
Phagocytosis and digestion	N.O.	+	++++	++++	N.O.
Extracellular destruction	N.O.	N.O.	++	N.O.	N.O.

N.O.—not observed.

Table III summarizes qualitatively the relative importance of various processes in the disposal of injected particles.

The author gratefully acknowledges the encouragement and helpful criticism of Dr. Leslie A. Stauber. Oysters were obtained through the courtesy of Dr. H. H. Haskin, New Jersey Oyster Research Laboratory, Rutgers, the State University. This investigation would not have been possible without the technical assistance of Kenneth Phifer and Sung Yen Feng. Dr. Ruth Gordon, Institute of Microbiology, Rutgers, the State University, kindly supplied the strain of *B. mycoides.*

SUMMARY

1. The fate of injected bacterial spores, vegetative bacteria and yeast cells injected into the oyster, *Crassostrea virginica,* was followed histologically and by viability studies.

2. Tissue sections showed that yeast cells were phagocytized in blood vessels by leukocytes which migrate into surrounding tissues and later through epithelial layers to the exterior. A small proportion of yeast cells remaining in the tissues are digested intracellularly.

3. Histologic examination of oysters injected with living bacteria revealed that the microorganisms were rapidly destroyed both intracellularly and extracellularly before significant numbers of host phagocytes could migrate to the exterior.

4. Viability experiments demonstrated that when several species of non-spore-forming bacteria were injected, very few of these organisms could be recovered 48 hours later. However, a small proportion of these bacteria persisted in oyster tissues for several days.

5. Bacterial spores were removed from oyster tissues at a much slower rate than vegetative bacteria.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

1960

ABSTRACTS OF SEMINAR PAPERS

JULY 5, 1960

Comparative biochemistry of connective tissue ground substance. MARTIN B. MATHEWS.

Structural proteins of the collagen type, along with neutral and acidic polysaccharides, possibly protein-bound, are typical components of connective tissue. Their conjoint distribution among animal species suggests a broad histogenetic pattern in evolution.

Intensive studies of mammalian connective tissues have led to characterization of a number of acid mucopolysaccharides which may be defined as linear chain acidic polymers with a disaccharide repeating unit containing glucosamine or galactosamine and galactose or an uronic acid. One or more ester sulfate groups may also be present. Acidic polysaccharides from invertebrate tissues are of different composition, with the possible exception of the mactins from clams which resemble heparin very closely. However, heparin (and precursors?) differs from the other mammalian acid polysaccharides in having an intracellular location and α-glycosidic bonds.

Preliminary results of a survey of vertebrates show predominantly chondroitin sulfate A isomer in notochord of lamprey and sturgeon, and predominantly chondroitin sulfate C isomer in cartilage of lamprey, shark, sturgeon and salmon. Keratosulfate is a minor component of these cartilages. The distribution from lowest to highest vertebrate forms suggests that the presence of chondroitin sulfate A and/or chondroitin sulfate C may be a systematic characteristic of vertebrate tissues.

Chondroitin sulfate C-protein complexes of shark cartilage were shown to be aggregates of basic molecular units which have a molecular structure and physical parameters nearly identical to the basic molecular units of chondroitin sulfate A-protein complexes of bovine cartilage. The relatively greater stability of the "C" complexes to heat and urea may reflect an adaptation to the high urea content of shark tissues. The remarkable similarity of molecular structure of "C" and "A" complexes indicates a corresponding similarity of physiological functions and suggests a common plan of molecular evolution of chondroitin sulfate-protein complexes, within which minor structural variations may occur.

Physical properties of muscle extracts. LEONARD NELSON AND W. F. H. M. MOMMAERTS.

Brief extraction of skeletal muscle fixed during rest, contraction or fatigue permits the investigation of changes in the configuration or interactions of the structural proteins consequent upon contractile activity. Paired sartorii, dissected from cold-room frogs and equilibrated in Ringer's solution for 30–90 minutes, at 0° C., were mounted on the electrodes of Mommaerts' quick-freezing apparatus. One muscle served as the resting control, while the other received 40 maximal stimuli in 2 seconds, in an atmosphere of cold moist N_2. During the height of contraction of the stimulated muscle, both were instantaneously frozen in isopentane at about − 160° C. The muscles were pulverized at liquid N_2 temperature, warmed to − 10° C., and extracted with ice-cold Guba-Straub solution. On dilution with four volumes of cold de-ionized H_2O, the resting muscle extract (RME) formed a voluminous flocculent precipitate which rapidly settled out, while the stimulated muscle extract (SME) invariably became opalescent, forming a fine particulate suspension only after several hours' delay. (However, if, during the preparation the muscles were equilibrated or stimulated at room temperature, the SME resembled the RME, suggesting that metabolic recovery processes may have intervened.) Centrifugation for 10 minutes, at 5000 × g. yielded 2–3 times greater volume of residue in the SME than in the diluted RME, although the total protein (Lowry method) was about 6–8% of the weight of the frozen muscle in both cases. Similar results were obtained from muscles equilibrated in solutions containing 1 mM. iodoacetate and 2 mM. lactate, one of the muscles stimulated to exhaustion at 0° C. (36 tetani). In a preliminary experiment, addition of 0.7 mM. ATP to aliquots of IAA-treated and extracted muscle caused formation of fine particulate precipitates in both resting and exhausted preparations within 10 minutes of dilution. Addition of 1 mM. creatine phosphate to other aliquots of the same preparations resulted in the appearance of large flocs in the RME and a 10-minute fine particulate in the SME. One may consider that after prolonged stimulation at 0° C., a substance such as ATP, or a precursor able to maintain ATP in the extract, may have been depleted below a critical level necessary to stabilize the actin-myosin interaction exemplified in the "super-precipitation" of the diluted resting muscle extract; while, at room temperature, biosynthetic reactions may restore this substance to the critical level.

July 11, 1960

A four-factor ionic hypothesis of spike electrogenesis. Harry Grundfest.

Potassium inactivation in electrically excitable membrane (*Prog. Biophysics*, **7**: 1, 1957) may be produced by alkali earth and onium ions, procaine and other agents, including inward (hyperpolarizing) or outward (depolarizing) currents. The chemical agents may or may not at the same time increase resting membrane resistance. Thus, pathways of resting and active potassium conductance may be pharmacologically different. Pharmacological agents also affect solium conductance mechanisms. Differences in type and degree of action of one agent in different cells, and of various agents in the same cell indicate that the varieties of electrically excitable membrane may be as diverse as are those of the electrically inexcitable (*Ann. N. Y. Acad. Sci.*, **66**: 537, 1957). While in many cells potassium inactivation is caused by inward currents and this gives rise to hyperpolarizing responses, in frog muscle fibers and eel electroplaques inactivation is caused by outward currents and results in anomalous rectification.

Operation of potassium inactivation (j), in addition to sodium activation (m), its inactivation (h) and potassium activation (n), permits explanation of phenomena that appear to be discrepant in the 3-factor theory. These are: conversion of arthropod muscles from graded to all-or-none responsiveness; properties of graded responses in eel electroplaques; the numerous varieties of prolonged spikes; effects of acetylcholine on cardiac spikes; change in K-electrode property; hyperpolarizing responses; anomalous rectification; passive resistance properties in spikes of frog axons and eel electroplaques; initial after-impedance in squid axons; oscillatory and "upside-down" potentials; various types of "stable states" in membrane potentials, and loss of delayed rectification.

July 12, 1960

X-ray-induced dissociation of the mitotic and micromere "clocks." Ronald C. Rustad.

X-irradiation of sea urchin gametes or zygotes can lead to the formation of micromeres at the first, second, or third cleavage rather than the fourth. Although cell division is delayed, micromeres are formed at the same time as the normal 16-cell stage. High doses, which induce multipolar spindles, usually obscure the effect, but occasionally micromeres are formed at the first multipolar division.

The biological clock which controls the time of micromere formation is independent of division *per se*, and of any event which occurs just once in each mitotic cycle.

The radiation-sensitive event normally occupies one-third of the mitotic cycle. Several workers have shown that the mitotic delay is expressed in this cytological stage. A hypothetical nuclear clock which operated continuously during interphase would lead to early micromere formation after x-rays because of excess interphase time. Similarly, a clock operating during the x-ray-sensitive phase would lead to early micromeres. A nuclear clock which ran during a non-delayed stage would lead to late micromere formation. The synchrony of micromere formation in control and irradiated zygotes suggests that the biological clock which controls the time of micromere formation is localized within the cytoplasm.

The giant cleavage spindle of the egg of Polychoerus carmelensis. DONALD P. COSTELLO.

This small acoel turbellarian is hermaphroditic, and after reciprocal mating, the eggs undergo fertilization and maturation within the body of each worm, advancing to the metaphase of the first cleavage division, where they come to rest with the chromosomes undivided. These eggs are up to 250 to 260 microns in diameter, if fixed at resting metaphase. The amphiastral spindle is a typical metazoan spindle, measuring up to 120 microns between the central bodies and up to 65 microns in diameter at the equatorial plate. All 34 chromosomes are J- or V-shaped, and arranged in a ring at the periphery with mantle fibers attached to the kinetochores. Continuous fibers fill the central spindle area. The centrioles are rods (straight or bent) up to 5 microns in length and one-half micron in diameter. They are usually oriented at right angles to the main axis of the spindle and, if one examines the centrioles at the two ends of any given spindle, at right angles to each other. This suggests that the centrioles of a resting spindle are "out of phase" with each other, and supplements some electron microscope observations of Porter (and of Burgos and Fawcett) indicating that recently divided centrioles in vertebrate material are cylinders at right angles to each other.

E. D. Hanson has recently revived a theory suggesting that the acoel Turbellaria originated from the ciliate Protozoa. If so, he suggests that the acoels should lack centrioles and should possess intranuclear spindles, like the ciliates. Such is obviously not the case, either in the egg, the blastomeres, or in the relatively rare division figures found in the parenchyma of young worms. Von Graff (1891) for *Monoporus rubropunctatus* and Gardiner (1898) for *Polychoerus caudatus* had early figured metazoan-type spindles in acoel eggs.

Aided by a grant from the National Institutes of Health, RG-5328.

Motion pictures of radiation-induced modifications in the early development of Arbacia. CARL CASKEY SPEIDEL AND RALPH HOLT CHENEY.

Graded exposures of gametes to either ultraviolet (2537 Å) or x-ray irradiation, with subsequent fertilization, were effective in bringing about deviations from the normal in developing sea urchins. These deviations included abnormalities in structure and delay in the developmental time schedule. Dosages ranged from very weak to very strong. The extent of radiation-induced injury, the amount of developmental retardation, and the maximal limit of differentiation and growth attained were all directly related to the dosage strength.

Illustrative cinephotomicrographs were obtained of the following features: (1) movements of intracellular constituents of irradiated eggs immediately before and after fertilization, (2) variations in the formation and elevation of fertilization membranes, (3) protoplasmic and granular movements during the first 90 minutes, culminating in normal and abnormal first and second cleavages, (4) aberrations in size and number of blastomeres in later cleavage stages, (5) initiation of twirling and swimming movements of blastulae, and (6) differentiation of gastrulae and plutei.

Time-lapse motion pictures gave a vivid portrayal of the radiation-induced changes visible in the early stages (1–4 above). Conspicuous viscosity changes were apparent in both the superficial hyaline layer and in the deeper protoplasm. After severe radiation the death throes of a zygote or of a later blastomere were frequently accompanied by accelerated internal upheaval. Normal rate pictures were more suitable for depicting the changes in the later stages (5 and 6 above). Crippled progeny included lopsided twirling gastrulae and misshapen plutei with abnormal patterns of arms, skeleton, and other features. There were both significant resemblances and differences in the various modifications induced by the two kinds of radiations.

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JULY 19, 1960

Electron spin resonance study of serotonin-FMN interaction. IRVIN ISENBERG.

The riboflavin semiquinone at strong acid pH gives a characteristic signal, using electron spin resonance techniques. If serotonin and riboflavin are mixed at pH less than one, a signal is obtained in spin resonance that looks similar to the riboflavin signal but has important differences. The similarity indicates that the riboflavin semiquinone is produced but the differences indicate that another free radical is also produced.

Previous spectrophotometric investigations have indicated that serotonin and riboflavin form a strong charge transfer complex. The present work may be interpreted as indicating that in strong acid solutions, the complex may dissociate into two free radicals, one of which is riboflavin. The other may be a serotonin free radical or some secondary free radical resulting from a reaction of the serotonin free radical.

Contractile responses in the presence of electron donors and acceptors. BENJAMIN KAMINER.

Isenberg and Szent-Györgyi have demonstrated that charge transfer complexes occur between electron donors and acceptors of biological importance. This led to an investigation on the effects of combinations of some electron donors and acceptors on smooth and striated muscle. In the case of a smooth muscle preparation (the proboscis of *Phascolosoma gouldii*) it was previously reported that preparations which did not respond to serotonin (a good electron donor) could be induced to contract by pretreatment with electron acceptors such as riboflavin, acridine red or sevron blue. Further investigations on striated muscle (sartorius of *Rana pipiens*) demonstrated that a good electron donor such as iodide (NaI having been substituted for the NaCl in Ringer's solution) or caffeine (10^{-3} M), could induce contracture, provided the muscle was pretreated with an electron acceptor such as iodine ($0.26–1.3 \times 10^{-4}$ M) or 1,2,-naphthoquinone (10^{-3} M); each substance alone, however, did not produce such an effect. The contracture varied in amplitude and, when marked, was usually irreversible and associated with a loss of response to electrical stimulation. It should also be noted that the iodide and caffeine have another similar action in that they both increase the twitch tension. Thus it appears that in both smooth and striated muscle, contractile responses are obtained with electron donors when in the presence of electron acceptors. These findings suggest the possibility that electron transfer could be involved in excitation of muscle. (This work was done during the tenure of a Rockefeller fellowship.)

Detection of electron donors. ANDREW HEGYELI.

Good electron donors, such as indole, give a strong colored charge transfer complex with iodine, which is a "local" electron acceptor. This phenomenon can be used in paper chromatography for the detection of good electron donors in tissue extracts. In order to localize the substances with good electron-donating properties on the chromatogram, the paper is exposed to iodine vapor. For this purpose we have a glass cylinder which contains, at the bottom, dishes filled with iodine crystals. Over them is a propeller driven electromagnetically. This distributes the iodine vapor uniformly.

JULY 26, 1960

The action of trypsin on acetylmyosin. W. ROBERT MIDDLEBROOK.

Myosin was acetylated by treating it with excess acetic anhydride in 1 M K_2HPO_4 at 0°. The resulting material was soluble in water, and stable to freezing and freeze-drying. Approximately 100% of the epsilon amino groups of lysine were acetylated. The material behaved similarly to myosin in the ultracentrifuge and separated into two peaks after digestion by trypsin, similar to heavy and light meromyosins. The digested acetylmyosin was depolymerized into sub-units with an average chain weight of 5000, on treating for 24 hours in 5 *M* urea. The N- and C-terminal groups were identical with those obtained from the meromyosins. Complete digestion of myosin and acetylmyosin by trypsin was followed in the pH stat. The difference between the asymptotic values of the two curves was equivalent to the lysine content of the myosin, and could be explained by the fact that acetyl-lysyl peptide bonds are not hydrolyzed by trypsin.

The structure of water in tissue, as studied by nuclear magnetic resonance. H. J. C. BERENDSEN.

A useful method for obtaining information about the water in tissue is the study of proton magnetic resonance. When a material containing protons is placed in a strong magnetic field, energy is absorbed in the radio-frequency range, due to the fact that the two possible spin states of the protons have different energies in a magnetic field. The shape of the absorption curve is determined mainly by the interaction and the motion of the protons, strong interactions and slow motion giving rise to broad absorption curves. If, in the study of hydrated macromolecules, oriented samples can be used, additional information is obtained by measuring the absorption as a function of the angle between the sample axis and the magnetic field.

The study of partially dried collagen showed that the classical picture of absorbed water does not hold, but that a considerable structure is present in the water. The best explanation of the experimental data is that in partially dried collagen the water molecules form chains in the fiber direction that rotate restrictively about an axis along the fiber axis. With the natural amount of water present, there is a faster exchange of protons.

A peculiarity of the three-fold collagen helices is that the axially repeating distance along the molecules equals exactly six second-neighbor distances of water, *i.e.,* a-axis repeats of ice (4.74 Å), thus stabilizing the existence of water chains by hydrogen bonds at the right places.

The building of a model showed that water probably can form cylinder-symmetrical structures with a repeating angle of 36° and an axial repeat of 4.74 Å. It is interesting that both deoxyribonucleic acid and collagen have repeating angles of 36° and axial repeats that are multiples of 4.74 Å.

A structural organization of water in tissue has implications for charge and energy transfer and for the theory of sodium extrusion as a bulk property.

Sponsored by the Research Laboratory of Electronics of the Massachusetts Institute of Technology, Cambridge, Mass.

Pinocytosis, phagocytosis, and lysosomes: cytochemical and electron microscopic studies. ALEX B. NOVIKOFF.

Cytochemical stains and electron microscopy permit the study of lysosomes, characterized by: (1) acid phosphatase activity in cell smears or frozen sections (fixed in cold formal-calcium) incubated in Gomori glycerophosphate medium, (2) a single ("unit") outer membrane. In erythrophagocytes, Kupffer cells, lung macrophages, cells of proximal convolution and glomerular epithelium, phagocytosis-pinocytosis vacuoles have been studied in both procedures and have both properties. Acid phosphatase activity is also present in *Chaos chaos* pinocytosis vacuoles, *Ameba proteus* food vacuoles, and vacuoles of spleen macrophages. The distinction between phagocytosis and pinocytosis seems less rigid: the vacuoles are bounded by former cell membrane and appear to have high acid phosphatase (and presumably other acid hydrolase) activities; as light microscopy visualized colloidal particles, electron microscopy now visualizes ferritin molecules in solution. Tracers (ferritin [glomerulus, Farquhar and Palade; *Trypanosoma mega,* Steinert and Novikoff]; colloidal particles [liver, Hampton; proximal convolution, Burgos]; enzymatic proteins—peroxidase, acid and alkaline phosphatase [kidney, liver, Novikoff *et al.*]) indicate that micropinocytosis vacuoles transport materials into larger vacuoles or lysosomes. To determine whether the small vacuoles also are lysosomes will require visualization of low levels of hydrolase activity in well-preserved electron microscope preparations. Awaiting future study are the origin of acid hydrolases, the relationships of "cytotic" vacuoles and Golgi apparatus, and the quantitative and qualitative significance of "cytosis" in the cell's economy. From their vital dye uptake and acid phosphatase activity (Pasteels, Mulnard, Dalcq), metachromatic granules appear to be lysosomes; from their fine structure (Rebhun) they appear to be "multivesicular bodies" (Sotelo and Porter). These bodies are seen in many cells; their unit vesicles might be formed by micropinocytosis or a membrane-generating process involving the Golgi apparatus.

AUGUST 1, 1960

Electrical inexcitability of the frog neuromuscular synapse. ROBERT WERMAN.

Frog muscle endplates were explored with an extracellular microelectrode. An intracellular electrode simultaneously monitored invasion into the endplate region of a spike which was directly evoked by a distant microelectrode in the same fiber. In the external recordings from sites generating miniature endplate potentials, an external positivity was invariably seen in association with the rising phase of the intracellular spike. Frequently, a subsequent small prolonged negativity terminated the potential. In contrast, when the exploring microelectrode was moved as little as 5 μ, the external recording became a positive-negative-positive sequence which is characteristic of the approach, local development and onward movement of propagated responses. The negativity was associated with the rising phase of the intracellular spike and its amplitude increased as the exploring electrode was moved further from sites of externally recorded miniature activity. It is generally agreed that the external potential closely follows the time course of the membrane current. Thus, the absence of negativity associated with the rise of the invading spike indicates absence of inward current at sites of externally recorded miniature activity. The late negativity can be accounted for by capacitative current through passive membrane. Since the sites at which the miniature endplate potentials are generated are presumably also the sites of synaptic activation, the synaptic membrane appears not to be excited by an electric stimulus of the magnitude of an action potential.

AUGUST 2, 1960

Free radical formation during indophenol blue synthesis by heart muscle respiratory enzymes. PHILIP PERSON AND ALBERT FINE.

A study of indophenol blue synthesis from dimethylparaphenylenediamine and alphanaphthol, as mediated by Keilin and Hartree type beef heart muscle preparations, has revealed that of the above reagents only the diamine is enzymatically oxidized by the heart muscle particulates. Enzymatic oxidation of the naphthol could not be detected using manometric and spectrophotometric methods capable of measuring such oxidations. The first step in the sequence of events leading to indophenol blue synthesis is the oxidation, by the heart muscle particulates, of the diamine to a free radical. The latter is stable and may be identified by its absorption spectrum which contains maxima at 550 and 520 mμ. The free radical forms a dimer, also identifiable by an absorption maximum at 640–650 mμ. Indophenol blue is formed as a result of a non-enzymatic oxidative coupling of the free radical and its dimer with the alpha-naphthol.

AUGUST 9, 1960

Cation permeability in muscle. R. A. SJODIN.

If the influx of potassium ions in frog sartorius muscle is determined at various external concentrations of potassium, the results can be fit to the "constant field" equation with a nearly constant permeability coefficient, as long as the system is close to the steady-state. When rubidium or cesium ions are employed, the influxes decline more with rising concentration than predicted by theory. When the potassium content of the muscle is not in the steady-state, the calculated permeability coefficients vary up to 15-fold, depending on the sign and magnitude of the driving force.

In general, the theory that ions in muscle are following simple electrochemical driving forces across the cell membrane appears inadequate and leaves one with the problem of accounting for the variations in measured permeability coefficients. One situation which could provide a basis for the observed departures from the flux equations is the case in which the number of membrane sites for a particular ion is limiting. A collision between an ion and an available membrane site seems a likely prerequisite for membrane entrance. The product of the external concentration and the number of sites available at the outer membrane surface should give a measure of the probability for membrane entrance from the external solution. This probability also involves a factor which represents the interaction energy (affinity) between the particular ion and the site.

It has been possible to demonstrate that the limited site model adequately describes the entry of potassium, rubidium, and cesium ions in frog muscle, either individually or competitively in mixtures. The affinity constant required for each ion remains invarient over a 50-fold range of concentration and flux. The order of decreasing affinity was found to be Rb > K > Cs.

Effects of ions on potential in lepidopteran muscle fibers. PETER BELTON.

Nerve-muscle preparations were made from the prothoracic leg of saturniid moths and the longitudinal locomotor muscles of pierid larvae. The effects of changes in external concentrations of K, Na, Ca and Mg on resting and neurally-evoked action potentials (r.p., a.p.) were described. The latter are graded events and are compounded from synaptically and electrically excited components. Changes in r.p. were evoked more readily by variation of K_0 than by the other three cations. A KCl solution isosmotic with the control saline did not depolarize muscle fibers immediately but prolonged the falling phase of the synaptic component. Longer soaking produced a neural block or repetitive firing of the muscle as a result of depolarization of the nerve terminals. K-free salines initially shortened the falling phase of the a.p. but later caused a plateau similar to that found in frog muscle with K- and Cl-free salines.

Omission of Na greatly reduced the electrically excitable and synaptic components of the response. When Na was replaced by sucrose, an initial enhancement of r.p. and a.p. was seen.

Seventy mM/l. Mg did not usually delay the take-off of the electrically excitable component, but decreased the size and increased the duration of the a.p. Neither did the omission of Ca block neuromuscular transmission. When responses were evoked at the rate of 0.5/sec. slight increases in r.p. and a.p. were evident in solutions with 30 mM/l. Ca. When paired stimuli were used, the relative refractory period for the electrically excitable component was found to have increased from 10 to about 500 msec. Bursts of stimuli at 50/sec. decreased the size of the responses more rapidly than stimuli at 25/sec. The effect of Ca_0 increase was more rapid than that of the other changes described.

It is concluded that other factors can, to some extent, override the K and Na electrode properties of the excitable membrane of these cells.

AUGUST 16, 1960

A scheme for the mechanochemisty of muscle. FRANCIS D. CARLSON.

Our studies on frog muscle have shown that 0.286 μmole/gm. of phosphocreatine are dephosphorylated in an isotonic twitch with a time constant less than thirty seconds. This corresponds to approximately one molecule of phosphocreatine split for each molecule of G-actin in the muscle. Chance and Connelly found only 0.009 μmole/gm. of adenosine diphosphate produced per twitch, and the half time of ADP rephosphorylation was of the order of minutes. The following scheme which reconciles these results assumes, as the stoichiometry cited suggests, that the reversible transformation of G-ATP-actin to F-ADP-actin occurs in the muscular contraction-relaxation cycle. Reaction one: G-ATP-actin is dephosphorylated during the contraction-relaxation cycle to give F-ADP-actin and orthophosphate. Reaction two: F-ADP-actin forms G-ADP-actin during depolymerization. Reaction three: G-ADP-actin is rephosphorylated directly by the creatinephosphokinase system as reported by Strohman, or else indirectly by exchange of traces of free ATP for the bound ADP and subsequent phosphorylation of the latter by the creatinephosphokinase system to give more ATP. These reactions involve nucleotide bound to actin and hence not available to exchange readily with free nucleotide. Reaction four: Phosphocreatine and ADP are formed in the sarcoplasm from the creatine-phosphokinase reaction as a result of the displacement of equilibrium by the creatine produced in reaction three. The equilibrium of reaction four favors creatine and ATP formation; hence only small amounts of ADP and phosphocreatine will be produced. Reaction five: Mitochondrial oxidative phosphorylation, stimulated by the ADP from reaction four, produces ATP which results in the restoration of creatine, phosphocreatine, and ADP concentrations. The slight increase in sarcoplasmic ADP produced by reaction four would correspond to that seen by Chance and Connelly. In the absence of glycolysis and oxidative phosphorylation the creatine and phosphocreatine concentrations would not be restored and our results would obtain.

Reversibility of actin depolymerization in presence of KI. ANDREW G. SZENT-GYORGYI AND EVA M. SZENTKIRALYI.

In presence of 1 mM $MgCl_2$ and 0.1 mM ATP or phosphocreatine (PC) a relatively high concentration of KI (0.8 M) is required for the complete depolymerization of purified F-actin. At lower KI concentrations the viscosity of actin has intermediate values. Depolymerization performed in presence of Mg and phosphagens can always be reversed by reducing the ionic strength. If Mg or the phosphagens are omitted, depolymerization is complete already at 0.3 M KI and is irreversible. The effect of PC and ATP can be explained by assuming the simultaneous polymerization and depolymerization of actin, a process similar to the findings of Oosawa *et al.* (*J. Polymer Sci.* 37: 1323, 1959) in low concentrations of $MgCl_2$. In accordance with such a picture, the steady formation of ADP, exceeding the stoichiometric ratio, can be measured at the intermediate KI concentrations, but not at 0.8 M KI where depolymerization is complete, or at 0.1 M KI where depolymerization is very slow. The maximum rate of ADP formation at room temperature occurs at 0.4 to 0.5 M KI, and 1 mole of ADP is formed in about 100 minutes by 57,000 grams of actin. The role of Mg in these reactions is specific and cannot be replaced by Ca, neither do ATP and PC act without Mg. During depolymerization G_{ADP}-actin is formed which requires Mg for its stability, possibly by facilitating the binding of the nucleotide to actin. When F-actin is depolymerized in the presence of Mg only, the resulting G_{ADP}-actin will not polymerize when the ionic strength is reduced, even though it is not denatured. Polymerization can be induced, though, by the addition of ATP or PC. The polymerization process thus can be separated into at least two steps. In the first step ATP or PC alters actin in such a fashion that it can polymerize, provided the ionic strength is favorable for the polymerization proper. During the process ATP is dephosphorylated. The nature of the intermediate is not known.

Studies on actin. II. Polymerization and the bound nucleotide. TERU HAYASHI AND RAJA ROSENBLUTH.

The necessity of actin for actomyosin contraction has been established, and the possible role of actin in muscle contraction as a localized, fixed site of phosphate turnover has been revived by Strohman and, more recently, Carlson. A crucial point in these speculations centers on the question—is the actin-associated nucleotide amenable to enzymatic attack while bound to the actin protein? The answer was sought in a study of the properties of G-ADP actin, since theoretically it was the form most likely to be the focal point for phosphate turnover.

At 1° C., G-ADP actin loses ca. 20% of its bound nucleotide in an hour, considerably faster at 29°. This loss is strongly inhibited by 1 mM Mg^{++}. It was found, surprisingly, that G-ADP actin is capable of polymerization upon the addition of salt; that is, it is not necessary to convert G-ADP to G-ATP for polymerization. However, at 1° C., G-ADP polymerizes slowly, whereas G-ATP polymerizes rapidly. In all cases, the extent of polymerization is directly proportional to the bound nucleotide. Thus, the facts have been established for the test of the principal question.

G-ADP actin in 1 mM Mg^{++} (where only traces of bound nucleotide are lost) is incubated with CP and CPase. The resulting solution is tested for polymerizability at 1° C. If the actin polymerizes slowly, it is still G-ADP, and no enzymatic transphosphorylation has occurred. If it polymerizes rapidly, it is G-ATP, and the bound nucleotide has been transphosphorylated. The latter is found to be the case, and it is concluded that the bound nucleotide of G-ADP is capable of enzymatic transphosphorylation.

August 23, 1960

The three hearts of the oyster. Paul S. Galtsoff.

The open circulatory system of the oysters and other bivalves is characterized by the presence of large sinuses and open lacunae or spaces which receive blood from the arteries. It is obvious that under such a condition effective circulation is impossible. This deficiency is compensated by several mechanisms: the pulsation of the radial vessels of the mantle; the squeezing of blood in the vessels of the gill lamellae by the contraction of gill muscles; the contraction of the wall of the common afferent vein; and by the contractions of the two accessory hearts located on the wall of the cloaca on each side of the adductor muscle. The accessory heart is not a simple tubular structure, as it had been heretofore described; it consists of three large branches, joined at a center in the form of a letter Y. Injection of dyes shows that the accessory hearts are in communication with the anterior pallial arteries, the renal sinus, the efferent veins and the circumpallial artery. It is interesting that the blood from the accessory heart enters this vessel against the ventricular pressure of the principal heart. The contraction wave starts from the center of the accessory heart and goes into two opposite directions. The rate of contraction is independent of the beating of the principal heart and is much slower. The function of the accessory hearts is to oscillate the blood within the pallium.

GENERAL SCIENTIFIC MEETINGS

AUGUST 29-31, SEPTEMBER 1, 1960

Abstracts in this section (including those of Lalor Fellowship Reports) are arranged *alphabetically by authors* under the headings "Papers Read," "Papers Read by Title," and "Lalor Fellowship Reports." Author and subject references will also be found in the regular volume index.

PAPERS READ

The pupillary mechanism in the toad. Philip B. Armstrong.

The isolated sphincter pupillae of the toad (*Bufo marinus*) can be stimulated to contract by the direct action of light. If maximal light constriction is produced and the illumination of the pupil continued, there is a slight adaptation to the light, as manifested by a slow continuing dilatation of the pupil. The constriction of the pupil produced by light is not antagonized by atropine even in high concentrations (10^{-3}). The threshold for acetylcholine is 10^{-9} with the dilated dark-adapted pupil. Acetylcholine 10^{-5} produces constriction about equal to that produced by maximally effective light. A further increase in the acetylcholine concentration (10^{-4}) results in some dilatation of the pupil but the sensitivity of the constricter to light appears to be unchanged. Acetylcholine is antagonized by atropine. Pilocarpine produced a slight constriction of the pupil but only in high concentrations, the threshold being 10^{-4}. Serotonin produces some slight pupillary constriction also only at high concentrations (10^{-3}). DL arterenol produces a striking dilatation in both the light-adapted and dark-adapted pupils. The threshold is 10^{-9} for the dark-adapted pupil.

Stimulation of the sympathetic trunk at the level of the most anterior vertebrae produces a striking pupillary dilatation, as does also the stimulation of a pair of nerves entering the eyeball at its posterior inferior aspect. It may be that this is a mixed nerve since occasionally a very slight constriction of the pupil can be induced by stimulation of these same nerves when the preparation is dark-adapted.

It appears that the sphincter pupillae has little if any motor innervation, that the dilator is sympathetically innervated probably by adrenergic fibers.

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A method for obtaining gametes from Asterias forbesi. A. B. CHAET AND R. S. MUSICK, JR.

A new method of obtaining starfish gametes has been developed, enabling one to obtain substantial quantities of fertilizable eggs (and sperm) even during the latter part of August. Animals were injected with an extract prepared from isolated radial nerves of large unripe starfish. The nerves, after washing in sea water, were lysed in distilled water for 10 minutes (1 cc. per nerve), and the supernatant was dialyzed against running sea water for 12 hours (or at 6° C. with frequent changes of sea water). A second treatment with distilled water yielded another fraction which also contained substantial concentrations of "shedding substance." Fifteen-hundredths cc. per gram of the heat-stable extract(s), which may be stored frozen, was injected through an arm into the coelomic cavity of a small, 10–30-gram (about 3-inch) ripe starfish. The animals were kept in dry iced fingerbowls (approximately 17° C.) until shedding was initiated, usually within 45 minutes. The eggs were collected in beakers by suspending the shedding females (oral side up) over beakers of sea water. Fifty to 70% of the animals injected in this manner shed, depending upon the state of the gonads. The eggs of 82% of the females shedding after an injection of the "shedding substance" exhibited 90% or better germinal vesicle breakdown. Preliminary evidence indicated that the eggs were highly fertilizable (88% or above). Yields of as much as 3–5 cc. of eggs were obtained from one 20–40-gram starfish. These small ripe starfish were obtained from cold water and were available until at least the latter part of August. Since the stock animals were stored in refrigerated sea water tanks, shedding could be induced when required. Further studies are being carried out on the characteristics and physiologic function of the shedding substance obtained from the starfish nerve.

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The modification of life span by x-rays for haploids and diploids of the wasp, Habrobracon sp. ARNOLD M. CLARK.

Haploid males, diploid males and diploid females of the wasp, *Habrobracon* sp. (an Indian species related to *Habrobracon juglandis*), show a decrease in adult life-span following exposure to x-rays as larvae-in-cocoons, white pupae or adults. The median life span for non-irradiated adults was 62 days for haploid males and diploid males fed on honey-water, 92 days for diploid females fed on honey-water and 40 days for diploid females fed on larvae of the Mediterranean flour moth, *Ephestia*. This difference in life-span related to sex, but not to genome number, indicates that the aging process is not due to an accumulation of somatic mutations during adult life. Haploid males exposed as adults to 10,000–50,000 r have a shorter life span than comparable diploid males. Diploid males and diploid females show similar decreases in life span relative to their controls. Pupae after exposure to 10,000 and 15,000 r and larvae after exposure to 2,000 r are equal to controls in post-embryonic survival and in ability to develop into structurally normal adults. These adults, however, show a decrease in life-span. Diploid males resulting from irradiated larvae or pupae show a decrease in life-span that is smaller than comparable haploid males but similar to diploid females. Radiation-induced decrease in life-span is markedly influenced by genome number but not by sex. This indicates that in contrast to the normal aging process, the decrease in life-span by x-rays is due to damage to the genetic material.

Distribution and substrate specificity of sea urchin egg phosphatases. GILLES COUSINEAU AND PAUL R. GROSS.

Phosphoesterase activities of sea urchin egg homogenates have been assayed against a variety of substrates and at three pH values. The substrates employed were glucose-6-phosphate (G6P), α-glycerophosphate (GP), nitrophenyl phosphate (NPP), fructose-1,6-diphosphate (F1,6P), and glucose-1-phosphate (G1P). These substrates were incubated with the enzyme sources at pH's of 5.4, 6.4, and 8.4. Fractions were obtained by differential centrifugation of the homogenates, which were prepared in isotonic KCl from unfertilized eggs that had been washed, successively, in filtered sea water, Ca-Mg-free artificial sea water, isotonic sodium citrate, and isotonic KCl. The homogenization medium contained 10^{-3} *M* EDTA. The expected activities for acid and alkaline phosphatases were observed. At pH 5.4, setting the hydrolysis rate for glucose-6-phosphate at 100, phosphate release rates for the other substrates were computed as: F1,6P, 132; NPP, 119; GP, 47; G1P, zero. At pH 8.4, with G6P again set at 100, release rates for the other phosphate esters were: F1,6P, 97; GP, 47; NPP and G1P, zero. At pH 6.4, where the activities of the acid and alkaline phosphatases could be expected to be minimized, significantly high hydrolysis rates were obtained for two substrates, G6P and NPP. The rate pattern was as follows: NPP, 415; G6P, 100; GP, 17; F1,6P, 17; G1P, zero. Although the behavior of these systems toward NPP at pH 6.4 constitutes a difficulty, the patterns of activity of the preparations are consistent with the existence, in the sea urchin egg, of a distinct glucose-6-phosphatase, possessing substrate specificities similar to those of the enzyme obtained from mammalian tissues. The homogenate fractions (pigment vacuoles, yolk particles, mitochondria, "microsomes," and soluble phase) showed different but characteristic patterns of activity against the substrates at the different pH values. The essential conclusions of this part of the study are as follows: the distribution of phosphatase activity among the fractions is different at the three pH values chosen, supporting the possibility that the catalysts are distinct from one another. Most of the glucose-6-phosphatase activity, so characteristically a "microsomal" function in mammalian cells, is localized in particles identical with, or sedimenting along with, the yolk granules.

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Osmolarities of some body fluids in the elasmobranch and teleost. H. Davson and Cynthia Thomas Grant.

Earlier work has shown that, in the mammal, the cerebrospinal fluid (CSF) is hyperosmolar to the blood plasma whilst the aqueous humour is approximately iso-osmolar; in the dog-fish (*Mustelus canis*) the aqueous humour is markedly hypo-osmolar to the plasma. In the present study on the dog-fish the osmolarities of the plasma, aqueous humour, CSF and the "subdural fluid" lying immediately below the cartilage of the skull were found to be 970, 947, 964 and 944 milliosmoles per liter, respectively. Thus, the CSF is approximately iso-osmolar with plasma whilst the "subdural fluid" is similar to aqueous humour. The chloride concentrations followed the same pattern, being 275, 245, 282 and 273 millimoles/kg. H_2O, respectively. In the scup (*Stenotomus versicolor*) the osmolarities of plasma and eye-fluids (a mixture of aqueous and vitreous humours) were 397 and 364 milliosmoles per liter; thus there is a high difference of osmolarity across the cornea of the teleost, but in spite of this the eye-fluids remain hypo-osmolar to plasma. It is likely that the "spectacle" represents an adaptation that slows down the osmotic withdrawal of water from the eye-fluids since removal of this skin from one eye caused a progressive increase in the osmolarity of its eye-fluids; thus, 1, 4, 6 and 10 hours after removal the differences between the two eyes were 10, 17, 31 and 40 milliosmoles per liter. The chloride concentrations followed the same pattern. In one fish it was found that its spectacle was opaque, presumably through damage to its epithelium, and it is interesting that the fluids of this eye were stronger than those of the other by 130 milliosmoles per liter. In *Tautoga onitis* plasma and eye-fluids contained 360 and 321 milliosmoles per liter, respectively. The intraocular pressure of dog-fish and scup was 10 cm. H_2O.

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The relationship between oxygen tension and light production before and after grinding up the light organs of fireflies. Arthur B. DuBois.

Fireflies of the local variety of Photinus and Photuris, when studied *in vivo*, require approximately 100% O_2 to achieve a maximal steady glow. Extracts of their light organs are said to require only 1% O_2 for a maximal steady glow. This difference may be due to a lower rate of reaction *in vitro*, requiring less oxygen, or to a diffusion gradient for O_2 *in vivo*, yielding a low O_2 tension at the site of reaction. The present experiment was an attempt to estimate the intensity of glow at different O_2 tensions before and after grinding up the light organ. A photomultiplier system was used to measure the light emission. A small glass tissue grinder was fitted with an air-tight rubber stopper and two side arms, to flush through different concentrations of O_2 in N_2 before or after grinding. Before grinding, the light intensity was easily measured, and increased up to a maximum of 20 scale divisions on pure O_2. After grinding, the light intensity was less than half a scale division (unreadable), even after adding ATP. The maximum intensity occurred at a concentration of approximately 10% O_2. It was concluded that the reaction occurred more intensely in the organized cell than in its homogenate, and hence more oxygen was used up in the intact cells, resulting in a larger diffusion gradient for oxygen. The reason for the decrease in light production after disruption of the cell structure was not determined.

Studies on actin. I. The properties of G-ADP actin. TERU HAYASHI AND RAJA ROSENBLUTH.

The changes of state of the muscle protein actin can be described as a cycle of events to regenerate actin; *viz.*, G-ATP polymerizes to F-ADP with KCl and Mg, F-ADP depolymerizes to G-ADP in water, G-ADP converts to G-ATP by two methods: (a) Incubation in water with ATP, or (b) incubation in small amounts of Mg with CP and CPase. Method (a) is known to be a whole nuleotide replacement; method (b) is of interest because of the possibility of transphosphorylation of the bound ADP. As a start toward a study of this possibility, a study of the properties of G-ADP as a key substance in the cycle was undertaken.

G-ADP polymerizes upon the addition of KCl, $MgCl_2$ and ATP. The extent of the polymerizability is a measure of the activity of the protein. At 1° C., 20% of this activity is lost in an hour, and is found to be due to the loss of bound nucleotide. At 29° the loss is faster. The continuous presence of Dowex-l, which quantitatively removes *free* nucleotide, also hastens the loss of bound nucleotide. Mg ion inhibits this loss to varying degrees, depending on the concentration of the Mg.

G-ADP also polymerizes without ATP, simply upon the addition of KCl and Mg. At 29°, this polymerization is similar to that produced with ATP, both in rate and extent, but at 1° it is much slower. A tentative explanation for this difference is given. Of considerable interest is the fact that it is apparently unnecessary to dephosphorylate the nucleotide for polymerization *per se* unless, in this case, the ADP is being dephosphorylated to AMP.

A procedure for obtaining completely dissociated sponge cells. TOM HUMPHREYS, SUSIE HUMPHREYS AND A. A. MOSCONA.

When sponges are pressed through bolting cloth, most cells remain in clumps. Further straining excludes larger clumps, but small clusters remain. This, as well as the likelihood of cell-type selection, and the variability inherent in this procedure, restricts its usefulness in studies requiring dispersed cells. An effective method, based on the removal of calcium and magnesium, was therefore developed for complete dissociation of sponge tissue into viable cells. It has been extensively applied to aggregation studies on *Microciona prolifera*, but is effective also for *Cliona celata* and *Haliclona permollis.*

One gram of blotted sponge lobe tips was immersed in 80 ml. calcium- and magnesium-free sea water (CMF-SW) at 0° C. (NaCl, 27.0; KCl, 8.0; Na_2SO_4, 1.0; and $NaHCO_3$, 0.04 gm./l. H_2O, pH 7.2), cut into 2-mm. fragments, soaked for 30 minutes, and squeezed through #25 standard quality bolting cloth into 80 ml. fresh, cold CMF-SW. The resulting suspension contained single cells and small clumps at an approximate concentration of 15×10^6 cells/ml. The material was centrifuged at 1000 rpm and resuspended at a concentration of 20×10^6 cells/ml. in CMF-SW containing 0.1% trypsin (crystalline-lyophilized, Worthington), at 0° C. Fifteen-ml. aliquots of this suspension in 100-ml. beakers were rotated on a gyratory shaker at 80 rpm for 6 hours at 5° C. Three ml. of the resulting suspension were then transferred into a conical centrifuge tube, vigorously squirted 15 times through a pipette ($\frac{3}{4}$-mm. orifice, 2-ml. bulb), spun down and resuspended in 1.5 ml. of sea water by pipetting.

The resulting suspension consisted of single cells. Maintained in stationary vessels, the cells formed aggregates which developed into sponges in a manner similar to that of conventionally dispersed sponge tissue.

It should be noted that trypsin did not noticeably assist in dispersion of the cells; however, for reasons yet unclear, trypsin-treated cells gave better aggregation results.

Rotation-mediated aggregation of dissociated sponge cells. TOM HUMPHREYS, SUSIE HUMPHREYS AND A. A. MOSCONA.

Aggregation of dissociated sponge cells provides a useful means for studying various cellular activities and interactions. Conventional "self-aggregation," in depending largely on cell migration and cell-sheet contraction, is susceptible to variables indirectly related to the pertinent problems, and is therefore inadequate for analysis of cell interactions. In the present procedure, cellular material is continuously maintained in suspension by rotation on a shaker and the cells brought in contact by the motion of the liquid. All experimental parameters are readily controllable and, under standard conditions, the results are consistent and amenable to quantitative assessment.

Aliquots of *Microciona prolifera* cell suspension (30×10^6 cells in 3 ml. sea water) dispensed into 25-ml. Erlenmeyer flasks are rotated for 24 hours on a shaker, ¾-inch diameter of rotation, 80 rpm at 25° C. The cells rapidly accumulate in the center of flasks and within minutes begin to cohere. After one hour all cells capable of cohesion are in small, irregular, loose clusters; these tend to fuse and reach final size at about 8 hours. By 24 hours the aggregates are round, compact, smooth, average 0.12 mm. in diameter, contain 1 to 2×10^5 cells, and are capable of development. Under these standard conditions, consistent, reproducible "aggregation patterns," defined by size distribution, shape and compactness of aggregates, are obtained. These are highly sensitive to experimental conditions, thus providing useful baselines for testing selected variables.

Using this procedure it has been found that: (1) aggregates are obtainable across a wide range of cell concentrations, (2) cells from different species have distinct aggregation patterns, (3) variations in dissociation procedure affect aggregation, (4) aggregation is temperature-sensitive in that cells remain dispersed at 5° C., although brought in contact by rotation, and form only small clusters at 10° C., (5) calcium is the only divalent cation sufficient for normal aggregation, (6) no cell cohesion occurs in the absence of divalent cations.

Delayed cleavage of fertilized Arbacia eggs after treatment with a "nitrogen mustard" or with formaldehyde. MILTON LEVY AND PEDDRICK WEIS.

Bis (beta chloroethyl) ethylamine (a nitrogen mustard gas) inhibits mitosis. The chemical action of the agent results in "alkylation" by replacement of active hydrogen by the alkylamine group with elimination of hydrogen chloride. The presence of two reactive groups allows the formation of cross-links between molecules, and seems to be essential for the biological effect of nitrogen mustards. Formaldehyde can also form cross-links.

Experiments (initiated in 1944) were done to define the actions of these agents on Arbacia eggs and sperm by following the progress of the first and sometimes the second cleavage. Egg sets and sperm were obtained by electrical stimulation, washed, and divided into batches. At appropriate times a reagent was added in 1/50 of the final volume. Treatments were terminated by sucking the supernatant off the settled eggs and washing to a nominal dilution of 2500-fold. Eggs were fixed and counted at intervals from each batch. To compensate for the variability among sets of eggs, the normal first cleavage time (55–68 minutes at 21°) of each set is taken as 100% and delays are given in terms of this time. For constant time of exposure the delay increases with concentration, but at 0.0009 *M* formaldehyde and 60-minute exposure the eggs cytolyze. The delay increases with time of exposure. Thus, using 0.00044 *M* formaldehyde for 10 minutes before fertilization produces no delay, but 15 minutes produces 13% delay and 60 minutes produces 75% delay.

Time of treatment in the cleavage cycle is very important. For the same treatment (5 minutes, 0.00072 *M* nitrogen mustard) the following data were obtained. Treatment before fertilization 75% delay, started just after fertilization 150% delay, 3 minutes later 115%, 12 minutes later 106%, 20 minutes, 40%, 24, 30, or 45 minutes no delay in the first cleavage but considerable delays in the second cleavage. The same treatment of sperm caused a 60% delay in the first cleavage of eggs fertilized with them.

Constancy of the pCO_2 in the ocean. W. F. LOOMIS AND W. F. LOOMIS, JR.

The carbon dioxide tension or pCO_2 of the ocean was studied under a variety of conditions. Using scuba, syringes were visually filled with bubble-free ocean samples taken at various depths down to 60 feet. After collection, all samples were placed on ice and taken directly to the laboratory where their oxygen tension was determined by the Winkler method and their pCO_2 determined by a new and direct method that does not involve pH. Duplicate samples were taken at various depths and localities from April to August, sites including the ocean, fresh- and brackish-water lakes and a salt marsh. The diurnal cycle was studied by taking measurements at 3 PM, 9 PM, and 3 AM, in both fresh-water and ocean sites. The findings were compared to oxygen determinations made by the Oceanographic Institution at stations between Woods Hole and Bermuda at all depths down to the bottom of the ocean (5140 meters).

The highest pCO_2 found was 1.43% atm. This occurred in a 9° C. exolimnotic layer 15 feet down in a fresh-water lake in April. Since fully aerated water has a pCO_2 of 0.03% atm., this finding contrasts with the fact that no pCO_2 above 0.13% atm. was ever found in the ocean. Oceanographic data indicate a layer 1000 meters down that is about 55% saturated with air and hence has a calculated pCO_2 of around 0.3–0.4% atm. This would appear to be the maximum pCO_2 of the ocean as the per cent saturation with air rises both above and below this deep sounding layer. Since the diurnal cycle was found to be less than 0.1% atm. at 10 feet of depth in the ocean, it can be concluded that pCO_2 is an ecological variable that varies widely in fresh-water lakes but is held extremely constant in the ocean. This finding confirms the view that the oceans form a remarkably constant *milieu exterieure.*

Carbonic anhydrase in the female reproductive tract of elasmobranch fishes. CECILIA LUTWAK-MANN.

Some years ago the author discovered the presence of carbonic anhydrase in the endometrial mucosa and placental tissue of various mammals, and in the uterine deciduoma of the rat. The occurrence of carbonic anhydrase was also established in certain parts of the oviduct of the egg-laying hen. These studies clearly demonstrated that carbonic anhydrase in the female reproductive tract depends upon hormones which control the function of the genital tract.

It was now decided to extend the study of the genital tract of certain elasmobranch fishes, at different stages of reproductive activity. The investigation was done on the vivparous-placental smooth dogfish (*Mustelus canis*), the viviparous-aplacental spiny dogfish (*Squalus acanthias*), and the oviparous skate (*Raja erinacea*). The genital tract of immature, or mature but non-pregnant, dogfishes showed none or negligible carbonic anhydrase activity. On the other hand, carbonic anhydrase was markedly active during early, and slightly less so in advanced gestation. The enzyme was localized in the lining and the wall of the uterus, the degree of activity being roughly equal to that found in the gills of these fishes. The oviducts and shell-glands were less active. In mature egg-laying skates the oviducts were the main site of carbonic anhydrase activity; the shell-glands were also active, and slight activity was demonstrable in the lining of the uterine folds. Practically no activity was detected in these organs in immature skates. Early dogfish embryos (4–7 cm.), yolk-sacs, or mature eggs of either dogfishes or skates were inactive. The ovarian tissue of these elasmobranchs was devoid of activity. In general, carbonic anhydrase values were highest in the tissues of the skate, and lowest in the spiny dogfish.

Reducing substances in blood of toadfish and catfish. PAUL FOLEY NACE.

It was apparent early in the investigation of alloxan diabetes in fish that pancreatic beta cell changes and blood Folin values followed a similar course. The use of the enzymatic Glucostat determination for glucose, in conjunction with the Folin-Malmros procedure for total reducing substances, confirmed this relationship for glucose but showed that another substance or substances participated even more markedly. Because the major concern of the investigation was the post-alloxan regeneration of the beta cells of the toadfish as compared to the non-regeneration in the catfish, it appeared necessary to examine these substances as possible factors in this species difference. In both species of fish, these substances accounted for most of the early change in Folin value. In toadfish, 60 minutes after alloxan injection, the Glucostat glucose had increased only 10 mg.% above control levels, while the total Folin reducing substances had increased nearly 100 mg.%. In man, Saifer found only 10% differences in non-diabetic patients and 20% differences in diabetics. The suggestion that alloxan or its compounds were important components was not supported by the course of the blood changes or by the observation that egg-bearing females failed to show the post-alloxan increase in Folin value. The relationship of alloxan to uric acid led to assay of this compound in catfish blood after alloxan, with failure to demonstrate a significant contribution. Since Fashena had shown the importance of glutathione and glucuronic acid in the "saccharoid" fraction of human blood, both test tube and chromatographic studies of these substances were made, using catfish blood. No significant increases were found. In the toadfish, blood fructose appeared constant through post-alloxan hyperglycemia. N.I.H. A-1129 (C3).

Measurements of volume and composition of the swim bladder gas of toadfish (Opsanus tau). George Polgar.

A pair of narrow lumen plastic tubes were sewn into both compartments of the swim bladder of unanesthetised toadfish through an abdominal incision. Their distal ends, sealed with caps, were left outside the abdomen after suturing the skin. Following this operation, and later at regular intervals, the caps were removed and, through a hypodermic needle, gas samples were withdrawn into syringes, the plungers of which were a Mylar-covered oxygen electrode and a Teflon-covered carbon dioxide electrode, respectively, for measuring the partial pressure of these gases. First the bladder was allowed to deflate to induce gas production. Subsequently the gas samples were reinjected into the bladder. The gas volume was measured before each analysis, with the fish in a water-filled pressure cooker serving as a plethysmograph. The volumes were calculated from Boyle's law. In seven fish, the initial oxygen concentration was between atmospheric (20.9%) and 49%, being higher in the anterior than in the posterior chamber in all but one fish. After deflation, a less concentrated oxygen was found in a small volume at first, but 18–48 hours after the operation, a peak was reached with a single maximum value of 86%. The changes of oxygen concentration were generally parallel in the two chambers. In one fish the carbon dioxide concentration rose in the anterior chamber prior to the rise in oxygen concentration. In another one, there was no such early peak. The concentration of carbon dioxide did not exceed 6% in any determination made on 7 fish, and the direction of its changes was usually the same as for oxygen.

These experiments indicate (1) that it is technically possible to sample both chambers of the swim bladder repeatedly under nearly physiological conditions; (2) some trends have been observed in the changes of composition and volume of the gases after deflation; and (3) a consistent difference was found between the concentrations in the two chambers.

An addition reaction of the α,β-unsaturated ether linkage of plasmalogens. Maurice M. Rapport.

It was unexpectedly found that lipids containing high concentrations of plasmalogen (derived from Asterias eggs and Mytilus (whole animal)) lose their unsaturated ether linkage on standing for several days at 20–24° in chloroform-methanol solution. This instability was not seen when the original chloroform-methanol extract was exhaustively washed with water. Lipids which had lost the unsaturated ether linkage fully retained their capacity to generate higher fatty aldehyde as determined by p-nitrophenylhydrazone formation. These properties indicate an addition reaction of the unsaturated ether producing a mixed acetal (or hemi-acetal if the adduct is water). The reaction was observed in only one of two extracts of starfish eggs but in all four of the extracts of mussel that were studied. In unwashed chloroform-methanol extracts, the unsaturated ether was relatively stable. It has been suggested that the function of plasmalogen may be related to its capacity to add water-soluble compounds at the activated double bond. While the observations made are consistent with this hypothesis, interpretation must await a more complete knowledge of the reactants.

Inhibition of fertilization of Asterias, Spisula and Chaetopterus eggs by Arbacia dermal secretion. Herbert Schuel and Charles B. Metz.

Upon brief (60-second) exposure to tap water the adult *Arbacia* releases a yellowish-green fluid called dermal secretion (DS), which inhibits fertilization of *Arbacia* eggs. The

DS appears to inactivate some egg substance that is essential for fertilization. In order to determine if this were a highly specific effect, the *Arbacia* DS was tested for action on fertilization in other species.

Eggs of the starfish *Asterias forbesi* did not fertilize in the presence of DS. Eggs washed from DS exhibited an almost complete loss of fecundity; *i.e.*, even when very high sperm concentrations (0.2%) fertilized few of the eggs. Exposure of eggs to DS subsequent to elevation of the fertilization membrane did not inhibit cleavage. Sperm washed from DS fertilized eggs as readily as sperm washed from sea water. These results are similar to those that have been obtained with *Arbacia* eggs and sperm.

In the presence of DS, fertilization of the eggs of the clam *Spisula solidissima* was inhibited because the sperm were killed. Eggs washed out of DS fertilized as well as the controls (the germinal vesicle broke down), but the DS-treated eggs did not divide. Exposure of eggs to DS after fertilization resulted in an inhibition of cleavage that could be partly reversed upon return to sea water. High concentrations of *Spisula* sperm (10%) absorbed and removed from solution the fertilization and cleavage inhibitors.

Preliminary experiments indicate that DS inhibited the fertilization of *Chaetopterus pergamentaceus* eggs. This may be the result of action on both the eggs and the sperm. Eggs exposed to DS after fertilization did not divide.

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Further studies on the antimitotic action of heavy water. WILLIAM SPINDEL AND P. R. GROSS.

The immersion of fertilized sea urchin eggs in sea waters reconstituted with excess deuterium results in delays in, or blockade of, mitosis and cytokinesis. D_2O concentrations up to 30–40% cause relatively simple delays in the cleavage cycle, although the incremental periods may be very long. Higher concentrations change the kinetics of cleavage as well, and permit smaller total numbers of eggs to enter mitosis. At concentrations higher than 65–70%, cell division is totally blocked. These effects are entirely reversible upon removal of the deuterium-enriched medium and replacement with sea water, except in cases where fertilized eggs have remained blocked in D_2O for several hours. The blockade is related to the mitotic cycle in general and not to nuclear events unique to the first cleavage cycle, for eggs can be blocked in the same way during cleavage cycles subsequent to the first. Reproduction and division of the cell centers, and perhaps of the genetic material, appear to continue during the blockade of karyo- and cytokinesis, since cells which have remained arrested in D_2O for long periods divide directly into multiple-celled forms when washed with normal water. The inhibition of fertilization itself is an effect upon the egg, not the sperm, since motility and fertilizing power are retained for significantly long periods after immersion of spermatozoa in D_2O. Current notions concerning the mechanism of antimitotic action by D_2O invoke changes in the normal hydrogen-bond cross-linking of cytoplasmic structure-forming macromolecules and particulates. In this connection, it has been found that the cytoplasmic "viscosity," measured by particle sedimentation, rises exponentially with increasing deuteration of the medium, reaching, in 95% D_2O, values more than sixteen times as great as those characteristic of sea water controls. This type of effect is also observed in fertilized eggs. The simple cleavage delay in 30% D_2O falls sharply in absolute length as the temperature is raised from 15 to 29° C. The effect of this is to make the delay a constant fraction of the cleavage interval in the temperature range indicated.

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Comparative studies of malic and glutamic dehydrogenases. CLAUDE A. VILLEE.

The studies of Kaplan *et al.* (*Science* 131: 392, 1960) have shown that lactic dehydrogenases from different tissues of the same animal may react at different rates with the several pyridine nucleotide analogs. In addition, lactic dehydrogenases from different animals have reaction rates with the several analogs in patterns which indicate evolutionary relationships: closely related animals have lactic dehydrogenases with similar patterns of reaction rates with these analogs, whereas unrelated animals have lactic dehydrogenases with markedly different patterns. To determine whether other dehydrogenases show comparable differences, and give patterns of reactivity that correspond to those for lactic dehydrogenases, tissues from a variety of invertebrate and vertebrate animals were homogenized and centrifuged in the cold, to sediment particulate matter. Aliquots of the supernatant fluid were tested for malic and glutamic dehydrogenase activity with diphosphopyridine nucleotide (DPN), 3-acetyl pyridine DPN (APDPN), 3-pyridine aldehyde DPN (PADPN), deamino DPN, and thionicotinamide DPN (TNDPN). Reaction rates were measured spectrophotometrically at the wave-length of maximum absorption of the reduced pyridine nucleotide or analog. Control cuvettes contained the complete system minus the substrate (malate or glutamate), to correct for reduction of pyridine nucleotides due to other enzymes and endogenous substrates. The patterns of relative reaction rates with the different analogs for malic and glutamic dehydrogenases are quite different. The rates with DPN and deamino DPN are generally similar but the enzymes tested reacted much more rapidly with the other three analogs than with DPN. The differences which characterize each tissue are based primarily on the rates with APDPN, PADPN, and TNDPN. Malic dehydrogenases of comparable tissues, *e.g.*, ovary, of related animals, *e.g.*, *Arbacia*, *Strongylocentrotus*, *Asterias*, *Echinarachnius* and *Thyone*, were more similar than those of different tissues, *e.g.*, ovary, testis, gut, and muscle, from the same species. Although the absolute patterns of reactivity are different for malic, glutamic and lactic dehydrogenase, evolutionary relationships are discernible on the basis of any of these.

Experiments with Ca^{45} in marine egg cells. FLOYD J. WIERCINSKI AND JAMES K. TAYLOR.

The egg cells of *Arbacia punctulata* and *Spisula solidissima* were used in various experiments with Ca^{45}. Egg cells were exposed to sodium citrate and EDTA, then Ca^{45} was applied. With ultraviolet light and x-ray Ca^{45} was present in the medium during the exposure. Also, cells were incubated in calcium-free sea water with known amounts of Ca^{45}. Samples of dried eggs and dried medium on planchets counted with a beta detector gave data that were in the range of the standard deviation.

Observations were made with the cells crowded in Ca^{45} solutions of 0.8 mm. thickness by means of a beta gas flow detector coupled to a rate meter and a continuous recording device. Under normal conditions there was very little, if any, uptake of Ca^{45}. Cell calcium is in equilibrium with that found in sea water. Long exposure to ultraviolet light, 150 Kr of x-ray, sodium citrate and EDTA showed varied uptake of Ca^{45} by a decrease in the counting rate.

Autoradiographic stripping film technique on whole mounts, sectioned eggs and ovaries of Arbacia showed the localization of Ca^{45}. Arbacia were grown in Ca^{45} sea water for ten days. Sections of the ovary showed that the immature eggs were rich in Ca^{45}. These data indicate that calcium is incorporated into the cell structure before the maturity of the egg cell. Silver grain counts were made on whole mounts of Arbacia eggs from the unfertilized stage to the blastula stage. A progressive increase in the number of grain counts per unit area on the jelly coat and the cortex had been observed during development. Staining techniques indicate that the cortex is probably a layer of calcium-nucleic acid complexes. Many slides have been prepared for further analysis.

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A function for the nucleolus. W. S. VINCENT AND E. BALTUS.

The nucleolus has long been associated with concepts of protein synthesis. Caspersson and Brachet recognized that the basophilia of nucleoli was due to RNA, and postulated that the RNA portion of the nucleolus might direct protein synthesis. Recent biochemical studies have shown that at least two types of RNA are required in the synthesis of protein. One of these is bound to the RNP granules of the cytoplasm (granule RNA); the other of the RNA's is "soluble," and has been shown to act as an activation agent for amino acids through the addition of terminal nucleotides.

Our analyses have shown that the starfish oocyte nucleolus contains an RNA fraction which adds (terminally) two cytidylic acid residues. This fraction, when exposed *in situ* to C^{14}-leucine, binds the leucine with an alkali labile bond. Attempts to extract this RNA by the cold *M* NaCl technique of Goldthwait resulted in the solubilization of 80–85% of the nucleolus. Part of the RNA contained in this fraction is tightly bound to the protein, the rest remains in solution after the protein is precipitated by dilution to 0.1 *M* NaCl. The leucine appears to be initially bound to the free RNA; upon further incubation the specific activity of the protein-bound RNA increases, while that of the soluble RNA decreases.

Analytical ultracentrifugation of the cold salt extract reveals the presence of a large homogeneous peak with a sedimentation coefficient of 4.5 S. The C^{14}-leucine is associated with the RNA attached to this protein.

The data reported here, and many other observations in the literature, are consistent with the following hypothesis: A major function of the nucleolus is the provision of "soluble" or "activation" RNA for use in the synthesis of cellular proteins. The "activation" RNA provided by the nucleolus is bound to a carrier protein, likewise nucleolar in origin; this protein is considered to be essential for the integrity of the RNA.

Senior Research Fellow, USPHS, and Chargée de Recherches du Fonds National de la Recherche Scientifique, Belge, respectively.

The "black" eye colors in Mormoniella. P. W. Whiting.

Many mutations have occurred changing dark brown wild-type eyes to brighter colors—red, orange, yellow, peach, white. *Very few* mutants have been found with eyes darker than wild-type. These "blacks" undoubtedly occur much more frequently than the records show, possibly as frequently as the brighter colors, but are difficult to detect. They have been divided into two groups—the *bk* blacks and the *eb* (ebony) blacks. The latter have no effect on scarlet (*st*) or on vermilion (*vm*), *st eb* and *vm eb* being scarlet and vermilion, respectively. Dahlia, a dark red, appears black in combination with ebony (*da eb*). The action of the *bk* blacks appears to be to prevent the development of the bright pigments, leaving the black pigments unaffected. Thus scarlet, having no (or very little) black pigment, is changed to white (*st bk*); vermilion, with intermediate amount of black pigment, becomes light lavender (*vm bk*) and dahlia, with much black pigment, becomes dark lavender (*da bk*). It is considered that the lavender chroma is due to a structural condition overlying black pigment, as in the feathers of Blue Andalusian fowl. Both the *bk* blacks and the *eb* blacks change orange and peach to white (*or bk, or eb, pe bk, pe eb*). It is expected that certain genetics tests now under way clarify the seemingly contradictory effects in the ebony combinations with the bright color genes. Irradiation of dahlia, orange or peach stocks should show more "black" mutants than irradiation of wild type, scarlet or vermilion. Homologies and linkage relationships are being determined. There are at least three loci, with multiple alleles at one of them. (Work supported under contract AT(30-1)-1471 with the U. S. Atomic Energy Commission.)

PAPERS READ BY TITLE

The ionic composition of squid photoreceptors. R. G. Adams and W. A. Hagins.

Though the electrical activity of many retinas has been studied, little seems known of the electrochemical basis of the currents and voltages so frequently seen. The squid retina is a favorable tissue for use in estimating the chemical composition of photoreceptors, since at least 90% of the retinal cellular bulk consists of the receptors and their cell processes. Opened squid eyes were dark-adapted and equilibrated at 10° C. in oxygenated, sulfate-free artificial sea water for 2–3 hours in the dark. The retinas were then freed from their scleras in dim red light, blotted and analyzed. They contained 79% water, 35% of which was accessible to inulin and is thus considered to be "extracellular." Allowing for the extracellular fluid, the mean ionic composition of the retinal cells was: K^+, 300 mM; Na^+, 110 mM; Cl^-, 140 mM. Since the artificial sea water contained 10 mM K^+, 430 mM Na^+, and 571 mM Cl^-, the Nernst potentials for the ions were: Na^+, 34 mV; Cl^-, −35 mV; K^+, −93 mV. The ionic composition of the cells of the squid retina is thus grossly similar to that of many other types of cells. However, the failure of agreement between the K^+ and Cl^- potentials implies that the cell membranes are not equally permeable to both ions, or else the receptors are not fully equilibrated after 3 hours in ASW. Nevertheless it is clear that electrochemical batteries are available in the photoreceptor membranes for production of electrolytic currents of the type seen in other cells, such as nerve and muscle.

Transport of nutrients in the holothurian Leptosynapta inhaerens. A. S. D'Agostino and A. Farmanfarmaian.

Several investigators have reported that the digestive tube of holothurians, although permeable to water in both directions, is completely impermeable to a variety of substances tested. These experiments, in addition to histological observations, have given rise to the hypothesis that the transport of nutrients in these organisms is largely or entirely mediated by amebocytes which occur abundantly in various tissues. The mode of nutrient transport in the echinoderms in general is, furthermore, in confusion.

To elucidate some aspects of this problem a number of experiments were carried out, using the holothurian *Leptosynapta inhaerens.*

The body wall was cut from a point just posterior to the mouth to a point just anterior to the anus. The anus was ligatured and the mouth cannulated. The preparation was then rinsed in filtered sea water and suspended in 5 ml. of the desirable isotonic solution. Under these conditions both the body wall and the digestive tube continue to exhibit peristalsis for 8 hours in sea water. Punctures in the gut wall could readily be detected and the preparation discarded when a suspension of neutral red in sea water was introduced into the digestive tube. Experiments involving the use of various indicators showed the pH of the esophagus, stomach and the intestine to be 6.8, 6.8, and 8.0, respectively. In a number of experiments 2000–3000 μg. of dextrose in sea water solutions were introduced into the digestive tract, and at time intervals the outside fluid tested for dextrose. The results of these experiments show that there is a passage of dextrose from the gut to the outside fluid at a rate of 16–17 μg./min. at 20°–23° C. Under these conditions 70–80% of the sugar appears in the outside fluid within two hours. When parallel experiments were carried out in isotonic $MgCl_2$ solution, in which the animal was relaxed, the rate dropped to 8 μg./min. These results indicate that contrary to earlier reports for other holothurians, the digestive tube of *Leptosynapta inhaerens* does allow direct passage of dextrose to the outside, and that mechanisms other than simple diffusion may be involved.

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Directional movements of the intertidal snail, Littorina littorea. Douglas G. Alexander.

Individuals of *Littorina littorea* are located within a specific intertidal region. When they are displaced above or below this region they move back into the preferred zone. Through a marking and recapture program the intertidal movements were recorded. Individual snails were marked with quick drying paint during low tide. After varying lengths of time subsequent movements were determined by triangulation.

With the exception of the forms displaced above the spring high tide level, all displaced forms moved in a direction which compensated for their displacement and brought them back into the normal zone of distribution. Individuals released within the zone of normal distribution displayed random movement at a reduced rate compared to the directional movements of displaced forms. This rate of movement within the zone of normal distribution became further reduced after a series of tidal cycles. The initial movement may have been due to the abnormal concentration of marked snails at the release point, and their subsequent reduction in movement may have been the result of their integration into the naturally existing population. Snails moved only when covered with water. Animals displaced above the tide level failed to move, indicating that the snails are inactive when exposed to the drying conditions of low tide. The forms displaced below normal distribution were placed upon a different type of sediment, consisting of fine silt. The trails indicated a rather direct movement toward the region of normal occurrence.

This work was part of the student training program of the Marine Ecology Course and supported in part by the Department of Zoology, University of North Carolina.

Observations on the ribosomes of sea urchins. William S. Allison and Eloise E. Clark.

Preliminary studies, conducted primarily by ultracentrifugal methods, were undertaken in order to determine conditions for isolating and stabilizing the ribosomes of the sea urchin. Unfertilized eggs and embryos in various stages of development (mid-gastrulae, 48-, 60- and 65-hour plutei) were chosen as the material to be used. Examination of both the crude extracts of cells and partially purified preparations of the fast sedimenting components revealed that the conditions required for the stabilization of the larger ribosomes of the sea urchin were not the same as those reported for other organisms. It has been found that the addition of magnesium to the extracting solvent is necessary for maintaining the structural integrity of the so-called 70S particles of *E. coli* or the 80S particles found in yeast and various mammalian tissues. This does not appear to be the case for extracts prepared from sea urchin eggs or embryos. When extracts of either unfertilized eggs or plutei were prepared in the absence of added magnesium (using 0.01 *M* phosphate buffer at pH 7.0, or with this buffer containing 0.002 *M* ethylene diamine tetraacetate), the schlieren pattern in the analytical ultracentrifuge showed a major component which had a sedimentation coefficient of 70–75S. When comparable extracts were prepared using either Tris or phosphate buffer and with varying concentrations of magnesium (from 0.001 to 0.1 *M*), the fastest component observed sedimented at 23–26S, indicating that the larger components had been degraded. Partial purification of the 75S component by means of cycles of alternate low- and high-speed centrifugation, followed by suspending the particles in a buffer containing 0.1 *M* KCl, led to the disappearance of the 75S component and the appearance of components which were observed to sediment at 43 and 24S. It is of interest that whereas the classes of particles from this organism were similar to those observed in other forms, the properties of stability were found to be somewhat different.

Regeneration of the cardiac stomach in Asterias forbesi. John Maxwell Anderson.

Starfishes that feed by everting the cardiac stomach continually place this delicate and essential feeding organ in hazardous situations; it would seem that ability to repair or regenerate the stomach in case of loss or damage would be of great survival value to the starfish. In *Asterias*, eversion can be induced by soaking the animal in $MgCl_2$ solution and then squeezing one or more of the rays. The everted stomach can be excised by cutting it across at the peristome and just below the pyloric stomach and severing its connections with the retractor harness. Study of a series of animals thus prepared and sacrificed at weekly intervals has provided the following information: (1) within three weeks, the integrity of the gut is re-established and the wall of the regenerated organ contains all the normal tissue layers; (2) the new stomach forms by upward growth of a sleeve of tissue from the peristome, making contact with the retractor nodule in each ray and eventually joining the pyloric stomach; (3) establishment of contact with the retractor nodule apparently induces formation of a pouch-like enlargement in the adjacent sector of the stomach—where contact is missed, as sometimes happens, no pouch is formed; (4) at three weeks, the wall of each pouch has begun to form a small but normal pattern of gutters and folds, with normal histological differentiation in the epithelium—multiflagellated cells with huge, dense, spindle-shaped nuclei over the folds, single-flagellated cells with granular, ovoid nuclei in the gutters; (5) between three and five weeks, the intrinsic retractor fibers invade the connective-tissue layer, growing downward beneath the branching gutters to form the normal corresponding pattern; (6) although the stomach is small and its normal specializations are present in only rudimentary condition, the starfish is capable of ingesting and digesting prey (amphipods, small snails) within the fourth to fifth post-operative week. The regenerating cardiac stomach provides valuable material for study of early stages in histological differentiation.

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A day-to-day relationship between oxidative metabolism and world-wide geomagnetic activity. FRANKLIN H. BARNWELL.

O_2-consumption of mud snails, *Nassarius obsoletus*, was measured continuously with Brown recording respirometers from June 19 to July 19, 1959, in Woods Hole, Mass. There was found to be a remarkable similarity between the day-to-day changes in mean daily metabolic rate and the form of the day-to-day changes in magnetic activity as measured by the international magnetic character figure, *C*. Throughout the period of study the metabolic activity on any given day, n, was clearly related to the intensity of magnetic disturbance on the first and second days thereafter, $n+1$ and $n+2$. This is indicated by the coefficients of correlation, -0.550 for the relationship to $n+1$ and -0.539, to $n+2$. Examination of other lag or lead relationships revealed little or no agreement between the two series. Twenty-nine coefficients of correlation for other temporal relationships of metabolic activity on day n to magnetic activity, from day $n-15$ to day $n+15$, had a mean value of -0.068 and standard error of ± 0.028. The coefficient of correlation for each day $n+1$ and day $n+2$ was significantly different from this population, $p < .005$. Such a correlation suggests that in rigorously controlled, constant conditions metabolic rate of the snails was reflecting a response to a pervasive factor related to solar, and subsequent world-wide geomagnetic, activity.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

Comparative electrophysiology of supramedullary neurons. M. V. L. BENNETT.

The supramedullary neurons (SMC) of *Lophius piscatorius* (angler fish) and *Tautogolabrus adiposus* (cunner) have been studied with microelectrode recordings. In both forms, a number of similarities were found to the SMC of *Spheroides maculatus* (puffer; *cf.* Bennett *et al., J. Gen. Physiol.,* 1959, 43: 159). Spikes could be evoked by brief stimuli to the spinal cord or cranial nerves, or by tactile stimulation of the skin. These indirectly evoked impulses arise in the axons, some distance from the soma, but subsequently invade the latter. An inflection on the rising phase of the spike recorded in the soma indicates delay at the axon hillock. The impulses are initiated by two kinds of depolarization arising in the axon. One is long-lasting, graded, and presumably of synaptic origin. The other is due to reciprocal excitatory connections between the cells. These connections are electrotonic, since hyperpolarization as well as depolarization is transmitted during intracellularly applied testing currents. As a result, when one cell fires, it depolarizes adjacent cells and all cells tend to fire synchronously. Thus, on indirect stimulation, with a near-threshold stimulus, all cells tend to fire, or not to fire, and in multiple discharges to a single stimulus all cells tend to produce the same number of spikes. However, the synchronization is less effective than in *Spheroides,* and frequently the number of spikes differed in different cells. The electrophysiological similarities found in the three forms indicate that the supramedullary neurons are homologous in all. However, the function of the SMC remains unknown. In *Lophius* the impulses of the SMC were shown to pass out the dorsal roots, but not the ventral. Thus, whatever their function, it is probably similar in *Lophius* and *Spheroides.*

Electron microscopic studies of the reflecting structures of elasmobranch and teleost eyes. M. H. BERNSTEIN AND T. S. DIETRICH.

Comparative observations have been made of the elasmobranch tapetum and teleost argentea to correlate their fine structure and reflecting functions. Electron microscopic preparations of three elasmobranchs: smooth dogfish (*Mustelus canis*), sting ray (*Dasyatis centrura*), and basking shark (*Cetorhinus maxime*), and a teleost (*Tautoga onitis*) were used. Histological observations were correlated with the electron microscopy.

All of the elasmobranchs examined were similar. The tapetum is formed by modified choroidal cells and covers the whole outer surface of the retina. Cellular processes containing melanin granules interdigitate in regular alternation with elongate crystals (presumably guanine) and their associated cytoplasmic extensions. In the light-adapted eye, the inner aspect of the crystalline plates is covered by one or more layers of melanin granules. In specimens dark-adapted for 12 hours, the melanin granules have migrated away from the retina so as to expose more of the crystalline material to the incoming light.

The angle between the crystalline plates and the optical axis approximates 45 degrees over much of the eye. In the area of the optic pit the plates are perpendicular to the axis. Toward the anterior portions of the eye, the crystals remain parallel to one another, but also become parallel to the incoming light. That is, a change is observed between the angle of the plates and the light axis. The orientation of the plates with respect to the light axis changes from 90 degrees at the optic pit to 0 degrees near the ciliary body. It has also been observed that the relative amount of crystalline material appears to be greater in the posterior pole of the eye.

The argentea of *Tautoga onitis* lies between the choroid and cartilagenous sclera. Bundles of parallel crystals lie in a connective tissue net. They lack the orderly relationship seen in the tapetum. This might be expected since the camouflaging function of the argentea does not require optically true reflection.

In addition to a confirmation of the above observations, work is now being done on the migration of elongated pigment granules in the retinal epithelium of *T. onitis* during dark adaptation.

The morphology of starfish spermatozoa. M. H. BERNSTEIN AND L. G. FEHRENBAKER.

Electron microscopic examination of thin sections of the starfish (*Asterias forbesi*) spermatozoa shows a roughly spherical, slightly flattened head composed of an acrosome, nucleus and mid-piece. The acrosome is a complex mushroom-shaped structure contained within a bowl-shaped depression, as demonstrated by J. C. Dan, with the inferior portion elongating into a sac. The acrosomal depression is defined by a strong osmiophilic membrane. The acrosome body is faintly osmiophilic and contains a central electron-dense star. The star has a spherical central region with seven projections lying in a common plane. A crescentic electron-dense mass surrounds the acrosome body in its equatorial region. Posteriorly, the limiting membrane of the acrosome is thickened into a plate which separates the narrow caudal extension from the main mass of the acrosome body. A dense osmiophilic knob is attached to the plate and hangs down into the posterior sac.

The genetic or chromosomal material of the sperm head forms a cup around the posterior aspect of the acrosome. The nuclear material is granular and electron-dense in character.

The crescent-shaped mid-piece in turn holds the nuclear material of the spermatozoan on its superior concave surface. The mid-piece appears to be a single ring mitrochondrion with a central hole for the insertion of the tail. The mid-piece is separated from the head by a thin line of moderately electron-dense amorphous material, which thickens to a large mass at the periphery of the head mid-piece connection.

The sperm tail is composed of a central core of two filaments surrounded by nine pairs of filaments. The central core is lost at the entry of the tail into the opening of the mid-piece, as the remaining filaments extend into the mid-piece in the region of the tail attachment to form the proximal or ring centriole of the mid-piece.

A common membrane encloses the whole spermatozoan. It fuses anteriorly with the acrosome membrane, which in turn splits to form two membranes on either side of the outer dense crescent of the acrosome. The sperm membrane continues over the nucleus and mid-piece and is reflected onto the tail.

Hexose and pentose utilization by mackerel erythrocytes. T. A. BORGESE AND JAMES W. GREEN.

The object of the present study was to examine the anaerobic metabolism of the mackerel red blood cell. A glycolytic pathway exists in these nucleated erythrocytes, as measured by the disappearance of glucose and the production of lactic acid at 15° C. and pH 7.6. It was demonstrated that the glycolytic quotient was dependent upon the level of phosphate in the suspension medium. When the phosphate concentration ranged from 13 to 80 μM/ml. suspension, with an hematocrit of 13%, the glycolytic quotient dropped from 0.47 to 0.30, respectively, suggesting that an increasingly greater dependence was being placed on respiration.

On the addition of IAA or KCN there resulted a decrease in the rate of glucose disappearance. Lactate production was virtually abolished with 10 μM KCN/ml. suspension (hematocrit 15%). IAA resulted in a reduction in lactic acid formation of about 64% relative to control suspensions.

The production of lactate from ribose appears to rival, and in at least one experiment exceeded, the production of lactate from glucose. The use of KCN in ribose supplemented suspensions also decreased the rate of lactate formation to approximately 70% of the control. With the control cells the rate of ribose utilization was best represented by a biphasic curve. The more rapid initial phase had a rate of 0.61 μM ribose used/ml. suspension/hr. The second phase was considerably slower, 0.12 μM/ml./hr. The hematocrit in this experiment was 15%. Ribose disappearance in the presence of KCN was not measured since additional experiments showed that cyanide interfered with the orcinol reaction for pentoses. The apparent stimulation of ribose used was not a real effect but simply a linear function of the KCN concentration.

The overall results suggest that a glycolytic pathway exists in mackerel erythrocytes, and that pentose is metabolized to lactate via a path comparable to the hexose monophosphate shunt, which is the main alternative to the glycolytic pathway of carbohydrate metabolism in animal tissues.

Nature and action of the fertilization inhibitor from Fucus. J. M. BRANHAM AND C. B. METZ.

Certain extracts of the brown alga, *Fucus vesiculosus,* inhibit fertilization and the fertilizin agglutination of echinoderm sperm. The chemical nature and mode of action of the inhibitor(s) have been further investigated.

Activity was found in the ethanol-soluble fraction of water extracts of the alga. The inhibiting material was precipitated with lead acetate and recovered from the precipitate. Esping (1957) concluded that such preparations contained polyphenolic substances. Color tests (F^{+++}, $K_3Fe\ (Cn)_6$, I_2, OH^-), precipitation reactions (heavy metals, $Cr_2O_7^=$, $(NH_4)_2\ MoO_4$, CaO, streptomycin, gelatin, agar, and in salt solutions), solubilities (soluble in methanol, ethanol, butanol, insoluble in xylene, acetone, ether), and nondializability suggest that the activity may be associated with tannins. Tannic acid (Merck) showed similar biological activity when tested on *Arbacia punctulata* gametes.

Fertilization inhibition resulted from an action on eggs, for sperm washed from inhibiting concentrations of the extracts were capable of fertilizing eggs, whereas membrane formation and cleavage (*i.e.*, fertilization) were irreversibly inhibited by exposure of eggs to *Fucus* extracts. If fertilized eggs were exposed during membrane elevation, incomplete membranes resulted and cleavage was greatly altered. Eggs with complete membranes cleaved normally in the extracts. Trypsin digestion (one hour in 0.05%) did not restore fertilizability (cleavage) to extract treated eggs, although trypsin treatment prior to exposure to *Fucus* extracts rendered eggs insensitive to the inhibiting action of the *Fucus* extracts.

Fucus extracts inhibited both fertilization and fertilizin agglutination of sperm (where applicable) in all species tested. Thus, the action of the agents is not specific.

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Endocrine control of the chromatophores of the zoeae of the prawn, Palaemonetes vulgaris. EDMUND S. BROCH.

Work done on the color adaptation of the zoeae of decapod crustaceans has indicated that the color responses differ substantially from those of the adults: specifically that the chromatophores respond directly to light and show no background response. An exception to this was a slight albedo response noted by Keeble and Gamble in second zoea of *Palaemonetes.* This was attributed to the extension of the eyestalk at that stage. It has been reported that chromatophores of first zoea do not respond to adult chromatophorotropins. There is no mention in the literature concerning retinal pigment movements in zoeae, as has been reported in adult decapods. Larvae of *Palaemonetes vulgaris* were reared in the laboratory through the eight zoea stages. Animals from one brood were used in each experiment, to insure similar stages of development and minimal genetic variation. The study was made only of the monochromatic red chromatophores which make up the secondary system, as defined by Keeble and Gamble. In all experiments conducted, the chromatophores were in a concentrated state when animals were on a white background and completely expanded on a black background, thus displaying a true albedo response. This response occurred from the time of hatching to the eighth zoea stage. First stage zoeae with sessile eyes responded as completely and rapidly to different backgrounds as did second stage zoeae with eyestalks. The red chromatophores on an excised thoracic section of a second stage zoea, kept in crustacean tissue culture medium, contracted upon addition of brain extract, expanded upon addition of abdominal nerve cord extract, and contracted when eyestalk extract was added. The distal retinal pigment of animals kept in darkness did not move to a dark-adapted state. These observations indicate that endocrine control of the chromatophores in the zoea of *Palaemonetes vulgaris* is comparable to that of the adult.

Magnetic field strength and organismic orientation. FRANK A. BROWN, JR. AND FRANKLIN H. BARNWELL.

Snails, *Nassarius obsoletus*, were observed as they emerged from a magnetic-south-directed corridor into a symmetrically illuminated field under alternating conditions of the earth's magnetic field alone and experimentally modified magnetic fields. The characteristics of orientation of the snails were observed during their first 3 cm. of free movement. The experimental magnetic fields consisted of a reversed direction of the horizontal component of the field, and with a series of 8 horizontal strengths: 0.04, 0.1, 0.2, 0.4, 0.8, 2, 5, and 10 gausses. For each experiment 5 snails emerged alternately in the earth's horizontal field of 0.2 gauss and in an experimental field, first in an ascending and then in a descending order of strength. Four such experiments were always run contemporaneously, and always between 9 AM and noon to obviate influence of the known daily rhythm in orientation. Fifty groups of four experiments were conducted, uniformly distributed over a period of exactly two synodic months. The experimentally reversed magnetic fields resulted in an altered mean path of the snails. When the strength of the horizontal component (H) of the reversed field differed by no more than a factor of 3 to 4 (0.2, 0.4, 0.8 and 2 gausses) from the earth's total field, F (*ca.* 0.6 gauss), there was a synodic monthly cycle of response with right-turning over *full moon* and left-turning the remainder of the month. For reversed fields with H differing by a factor of 4 to 15 from the earth's F (0.04, 0.1, 5 and 10 gausses), there was similarly a synodic monthly cycle of response but now 180° out of phase, with right-turning over *new moon* and left-turning the rest of the month.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-1228-03.

A relationship between photic and magnetic response in snails. FRANK A. BROWN, JR. AND ANNICK HUTTRER.

Mud-snails, *Nassarius obsoletus*, were permitted to emerge from a magnetic-south-directed corridor over a polar grid such that the mean angle of right or left turning could be ascertained during their first 3 cm. of free movement. The conditions under which the snails emerged were the following, in order: (1) in the earth's magnetic field into a symmetrically illuminated white field, (2) in a field black to left and white to right, (3) same as the preceding but in a 5-gauss horizontal magnetic field oriented in reverse to the earth's, (4) same as (1), (5) in a field black to right and white to left, (6) same as 5 but in a 5-gauss horizontal magnetic field oriented as in (3). For each experimental run the series was repeated, with 5 snails in each condition, four times. Such experiments were carried out 18 times, 6 during mornings 8–10 AM, 6 during afternoons 1:30–3:30 PM, and 6 during evenings 6–9 PM. Incident illumination in all cases was 150 lux from above, 20 lux from the black side and 60 lux from the white. The snails showed a distinct positive phototaxis; when the black field was to the left the mean path was $+15.9 \pm 1.96°$, and when to the right, $-18.4 \pm 2.52°$. The deviation in path in response to the asymmetrical light field varied from experiment to experiment. There was, however, a distinct correlation between the strength of the response to the asymmetrical light field and the effect of the 5-gauss magnetic field. For weaker light responses ($< 15°$), either + or −, the magnet produced left-turning and for stronger light responses (15° to 42°), right-turning. The coefficient of correlation between strength of light response and strength of magnet response was $+0.40$ ($t = 3.6$, $N = 72$, $P < 0.001$).

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-1228-03.

A "compass-direction effect" for snails in constant conditions, and its lunar modulation. FRANK A. BROWN, JR. AND H. MARGUERITE WEBB.

At the same time each day, to avoid the daily rhythm of mean path of orientation, *Nassarius* were allowed to emerge from narrow corridors directed in four compass directions, North, East, South and West, into a symmetrically illuminated field. In four contemporaneously conducted experiments a total of 40 snails emerged in each direction between the hours 1 and 4 PM. For each of two synodic months there was minimum left turning, or maximum right turning when the snails were emerging northward. An analysis of the data indicated, however, that there was a synodic monthly modulation in the form of the change in mean path with compass direction. For both months, from first to third lunar quarter the relationship was bimodal with two peaks of greater right turning, one for the north- and the other for the south-directed snails, and for the semimonth from third to first quarter there was a unimodal pattern of fluctuation with maximum right turning in northerly directed snails and maximum left turning in southerly directed ones. The ranges of differences for the two semimonths following new moon (3.4° and 3.8°) were only about half those observed for the two semimonths following full moon (7.2° and 7.4°). That magnetic field clearly plays some role in this organismic "direction-effect" was evident in comparing the relative effects of reversing the field of the north- and south-bound snails with, consecutively, 0.4 and 2 gauss horizontal fields. For the north-bound snails the response to the weaker field lay 2.54° to the *left* of that for the stronger field, and, to the contrary, for the south-bound snails the response to the weaker field lay 2.92° to the *right* of that of the stronger field.

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Some effects of lysergic acid diethylamide and related agents on embryonic heart rate in Fundulus. JOSEPH A. BURKE.

In an attempt to ascertain the earliest measurable effect of lysergic acid diethylamide (LSD-25) and related psychotomimetic drugs on some specific organ, 25 *Fundulus* eggs were grown in 50-ml. solutions of lysergic acid diethylamide, 1-methyl-lysergic acid butanolamide (UML-491), yohimbine and serotonin, respectively, in sea water held in finger bowls at 21° C. In concentrations of 100, 50, 10 and 1 μg./ml., LSD-25 and yohimbine depressed heart rate. In LSD-25, development of a functional heart was delayed at least 48 hours. When a pulse was obtained in test embryos it varied inversely with concentration of drug. For example, with LSD-25, the first rates, after a functional heart was present in test embryos, were: control, 122 beats/minute; 100 μg./ml. LSD-25, 80/minute; 50 μg./ml. LSD-25, 97/minute; 10 μg./ml. LSD-25, 115/minute; 1μg./ml. LSD-25, 140/minute. Mean heart rates for all days up to hatching were: control, 147 beats/minute; 79, 93, 115 and 141 beats/minute for the 100, 50, 10 and 1 μg./ml. concentrations of drug, respectively. LSD data plotted as logarithm of dose against heart rate form a straight line. Using the least squares method, the resulting prediction equation is: heart rate equals 144 minus 32 times logarithm of dose. With yohimbine, mean atrial heart rates for 100, 50, 10 and 1 μg./ml. concentrations were, respectively, 102, 122, 131 and 152 beats/minute; control, 147. In yohimbine concentrations above 10 μg./ml., frequent atrioventricular dissociation, with a ratio of one ventricular beat to from 2 to 8 atrial beats, occurred. UML-491 had little effect on pulse rate. The effect of LSD-25 and UML-491 on embryonic heart rate apparently is unconnected with their antiserotonin activity: UML-491 has an antiserotonin activity over four times greater than that of LSD-25. Serotonin itself had no effect on pulse rate. The Sandoz Pharmaceutical Company kindly supplied the LSD-25 and UML-491.

Supported by National Institute of Mental Health grant MY-3235.

Some morphological effects of lysergic acid diethylamide and related agents on early embryonic development in Fundulus. JOSEPH A. BURKE.

To ascertain whether representative psychotropic drugs have an effect on development, fertilized eggs of *Fundulus* were exposed at 2- to 4-cell stage (Oppenheimer, stages 3 and 4) to 100, 50, 10 and 1 μg./ml. sea water solutions of lysergic acid diethylamide (LSD-25), methysergide (UML-491 Sandoz), yohimbine and serotonin. Twenty-five eggs per 50 ml. solution were grown in finger bowls at 21° C. The concentrations were low enough to obviate changes in osmotic pressure or pH. No inhibition was noted in stages from Oppenheimer 4 to 16: up to the stage when the forebrain expands to form the optic vesicle. At this stage all four substances exerted some degree of growth inhibition. LSD-25 was the most potent. In embryos in the 100 μg./ml. LSD-25 solution, heart formation was delayed for about 48 hours. No melanophores or xanthophores were visible; optic vesicles were small and often solid. The embryo was undersized and underdeveloped. The same effects were evident but to a lesser degree in the 50, 10 and 1 μg./ml. solutions. Hatching was inhibited in the higher concentrations; the embryos finally died if not removed from the LSD-25 solutions. In the UML-491 test embryos, there was similiar inhibition but a lesser degree: the embryos were smaller than controls; there were fewer melanophores and those were dispersed. Eventually the embryos hatched with controls, but such embryos remained slightly undersized. Heart formation was delayed about 24 hours. Serotonin in the concentrations used showed slight inhibitory action. Heart formation was not delayed; embryos in general were smaller. Yohimbine concentrations above 10 μg./ml. induced abnormal heart formation. Embryos in this substance, if hatched, soon died. Rated in order of potency for inhibition: LSD-25, yohimbine, UML-491 and serotonin. The Sandoz Pharmaceutical Company kindly supplied the LSD-25 and UML-491.

Supported by NIH grant MY-3235.

Dactyl chemoreceptors of brachyurans. J. CASE, G. F. GWILLIAM AND F. HANSON.

The presence of chemoreceptors sensitive to amino acids is demonstrated on all limbs of *Libinia emarginata, Callinectes sapidus* and *Carcinides maenas* by recording from small bundles dissected from the leg nerves during application of localized stimuli to various parts of the limb. These receptors, whose end organs are not known with certainty, respond well to boiled or dialyzed-aqueous extract of Mytilus, are but slightly stimulated by 25% and not at all by 200% sea water, and are unaffected by dilute hydrochloric and acetic acids. Methyl, ethyl, n-butyl and n-propyl alcohols at concentrations as high as 0.2 *M* are non-stimulatory, as are maltose at 0.5 *M* and lactose at 0.25 *M*. The polypeptides glutathione and tryptamine are non-stimulatory at 0.1 *M*. All amino acids tested were stimulatory, but of these dl-methionine, l-glycine, l-arginine, β-alanine, l-proline and l-lysine were active only at concentrations of 0.2 *M* or greater. The most active substance tested was l-glutamic acid with a response threshold approximating 0.001 *M*, either unbuffered or at pH 7.8, approximately that of sea water. Considerable specificity of the glutamic acid response is suggested by the poor stimulatory action of α-methyl glutamic acid and glutamine. Behavioral experiments in which these substances were applied to mouthparts and legs of *Carcinides* confirm these observations. An active compound applied to the mouthparts produces feeding movements or, if applied to a cheliped, causes immediate feeding movements followed by touching of the cheliped to the mouth.

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Secretory structures in the tube foot of starfish. A. B. CHAET AND D. E. PHILPOTT.

The means by which starfish manage to climb up the vertical side of a glass aquarium is usually explained by the suction cup theory. However, if tube feet adhered to a glass wall are cut, they will continue to remain in place even though the vacuum has been released. It has also been observed that the tube feet leave behind a stainable substance, as well as particles which can be observed under the electron microscope. With these preliminary observations in mind an attempt was made to study the ultra structure of starfish tube feet.

Tube feet of *Asterias forbesi* were obtained by allowing them to adhere to a glass surface, and then cutting the foot at its proximal end. They were fixed in osmic acid sea water and, after dehydration, embedded in methacrylate or vestopal. Observing both longitudinal and cross-sections in the electron microscope disclosed that the side of the tube foot, which contains at least two types of secretory glands and is covered with micro villi, is completely different from the base of the tube foot—the area which adheres to surfaces. The base of the tube foot contains many elongated tubes, each one possessing dozens of ellipsoidal structures appearing to be "secretory packets." Each "secretory packet," about ½ × ¾ microns in size, is surrounded by a layer of fine granules, inside of which may be found 50 or so fibers (200 Å diameter), arranged much like the continuous fibers of mitotic spindles. It has been shown that the "secretory packets" are secreted through the walking surface of the tube foot. It is possible that they function in "gluing" the tube foot to either rough or smooth surfaces, thus aiding in locomotion. Identical structures have been found in *Asterias vulgaris*.

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Recovery of uricase activity in concentrated urea solutions. Aurin M. Chase and Jean M. Bussard.

The oxidation of uric acid by uricase is inhibited in the presence of urea (Chase, 1957, *Biol. Bull.*, **113**: 320), although the experimental results can be greatly influenced by the kind and concentration of buffer used to stabilize the pH (Chase, 1956, *Biol. Bull.*, **111**: 299).

In addition to this immediate effect of urea, which (in the case of lower concentrations) is reversible upon dilution, a slower—relatively irreversible—inactivation of the enzyme (Worthington purified uricase) can also be demonstrated. This was observed when the enzyme was allowed to stand in 3 to 8 *M* urea solutions in 0.1 *M*, pH 9 borate buffer. After the uricase had been exposed to the experimental urea solutions for the desired time, samples were diluted ten-fold with buffer containing uric acid, and the initial rate of decrease of absorbance of the reaction mixture was measured at 290 mμ. The temperature of all solutions and of the spectrophotometer's cell compartment was maintained at 26° C. The rate of irreversible inactivation of the uricase was greater, the higher the concentration of urea to which the enzyme was exposed before dilution.

In addition to the reversible and irreversible inactivations just described, another effect of urea upon the enzyme was observed. At the highest urea concentrations studied (6 to 8 *M*), the uricase activity—as measured after the ten-fold dilution—increased rapidly during the first fifteen minutes or so in the undiluted urea solutions, and then decreased again slowly. This initial recovery of activity was relatively slight in 6 *M* urea solution but very pronounced in 7.5 or 8 *M* concentrations.

So far, such experiments have been performed only at 26° C., using borate buffer of pH 9. The effects of temperature, of pH, and of other buffers should obviously be investigated.

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Effect of non-static conditions during gamete irradiation on Arbacia fertilization and injury. Ralph Holt Cheney and Carl Caskey Speidel.

Fertilization membrane characteristics and injury expressed by developmental stages are determined *to a degree* by the state of motion during gamete irradiation. Gametes were exposed to 2537 Å ultraviolet (UV) rays at a constant distance while in a static state or while agitated by two-per-second oscillation excursions over one-half inch right and left from the stationary center. Shaken or non-shaken eggs after irradiation (4 seconds to 16 minutes) were inseminated with normal sperm. Membrane characteristics, developmental injury, and death were noted comparatively over a three-day period.

Four-minute UV-rayed shaken eggs produced concentric tight fertilization membranes. Non-shaken UV-rayed eggs, as a result of differential cytoplasmic damage, developed eccentric membranes. Such membranes usually assumed normal form some time prior to first cleavage. Normal eggs, fertilized by shaken or non-shaken sperm, showed no membrane eccentricity. Later abnormal stages were similar whether derived from irradiated sperm or eggs. Cultures from irradiated shaken eggs exhibited earlier and greater percentages of death than cultures from irradiated non-shaken eggs. This greater damage under non-static conditions probably was associated with the fact that injury depended upon depth of nucleus from the surface during irradiation. Shaking assured more uniform damage by bringing more egg nuclei into position for maximal reception of the UV rays.

Shaken or non-shaken x-rayed (2 to 120 kr) eggs did not give rise typically to eccentric membranes, but tight membranes resulted from strong doses. Deep x-ray penetration caused equivalent effects regardless of motion, whereas shallow UV-ray penetration induced differential effects.

Significant sequences of fertilization membrane formation and adjustments were recorded by time-lapse cinephotomicrography.

This investigation was supported by a research grant (PHS RG-4326 C3) to C. C. S. from the National Institutes of Health, Public Health Service.

Monoauxenic culture of Arcella vulgaris. ANNA CICAK AND J. B. WITTENBERG.

At the turn of the century Engelmann captured a few individual protozoans which formed indubitable gas bubbles in their cytoplasm. Bles, 20 years later, made an extended study of gas bubble formation in one of these forms, *Arcella vulgaris.* The nature of the gas is not known. In order to carry further the study of gas secretion by the organism, viable, densely populated cultures are essential. Although Arcella is commonly maintained in infusion cultures, the population density is low and the majority of the animals are in an unsatisfactory physiological state. We have found that Arcella maintained in a simple salt solution will reproduce vigorously when fed a suspension of washed *Aerobacter aerogenes* every few days. The Arcella remain attached to the bottom of the Pyrex dish. Spent medium is poured off and replaced once a week. The cultures have not as yet been obtained free of other organisms. However, at this stage the cultures contain a very dense population of vigorous individuals, many of which are seen to be dividing at any one time.

Development of the Ilyanassa embryo after removal of the mesentoblast cell. ANTHONY C. CLEMENT.

The mesentoblast cell (4d) of the molluscan egg gives rise to the primary mesoblast bands and a portion of the entoderm. Within a period of about an hour after its appearance, the 4d cell of the Ilyanassa egg can be experimentally removed by puncturing or tearing it with a glass needle; following injury the cell swells and separates from the embryo, without damage to neighboring cells. The development of about a dozen embryos of *Ilyanassa obsoleta* has been followed after removal of the 4d cell. In the better cases, normal or nearly normal development has been observed of velum, eyes, cerebral ganglia and commissure, foot, operculum, statocysts, stomodeum, esophagus, and shell. The larval retractor muscle was seen in one case. The shell may give the appearance of being empty, except for the mantle and occasional masses of yolk spherules. The esophagus leads into an entodermal complex which is situated in front of the shell, and in which components of the liver and stomach can be recognized. Neither intestine nor heart has been observed to develop after removal of the mesentoblast cell. Absence of the intestine accords with Conklin's conclusion that this structure arises (in Crepidula) from the entodermal derivative of 4d. The larval heart of prosobranchs has been described as an ectodermal vesicle which pulsates by means of mesodermal muscle cells. The present experiments suggest that the heart musculature is derived from the 4d cell.

Rectification in skate electroplaques and its abolition by barium ions. B. COHEN, M. V. L. BENNETT AND H. GRUNDFEST.

The electroplaques of *Raja erinacea* and *R. ocellata* exhibit a marked increase in conductance when they are depolarized by more than 10 mV. The conductance change tends to restore the membrane potential to about 5 mV positive with respect to the resting value. The change requires one to several msec. to develop. It decays over several hundred msec. after a brief initiating pulse, and it is graded, depending on stimulus strength and duration.

The conductance increase occurs predominantly in the uninnervated, caudal face. It is qualitatively similar to the "delayed rectification" in squid axons, which has been attributed to increased K^+-conductance, and is a mechanism which increases the external current flow that is produced by the discharge of the electrically inexcitable innervated membrane. Though it is potential-determined, *i.e.*, electrically excitable, delayed rectification is not regenerative. Presumably, it lacks the component of inward flux of positive charge which provides the regenerative property of spike-generating membrane.

On applying Ba^{++}, the rectification was eliminated with little change in resting resistance, or in the responses to ionophoretically applied acetylcholine. In addition, there was a small depolarization. The neurally evoked responses were elicited for some time after the delayed rectification was eliminated, but the presynaptic fibers soon became inexcitable, often passing through a stage of repetitive firing, as indicated by "spontaneous" depolarizing potentials in the electroplaques.

Thus, the electrically inexcitable membrane component which produces the neurally and chemically evoked depolarization of the electroplaque was not affected by Ba^{++}, while the electrically initiated processes that produced the delayed rectification were blocked. The effect on potential-determined processes may be related to the block of K^+-conductance by Ba^{++} and other agents in various electrically excitable cells (Grundfest, this issue).

Permeability of red blood cells to alloxan. S. J. COOPERSTEIN, DUDLEY WATKINS, EVELYN HALPERN AND ARNOLD LAZAROW.

Previous studies on the comparative distribution of injected radioactive alloxan, urea, mannitol and inulin in the various tissues of the toadfish suggested that alloxan may not enter the cell; it is possible, therefore, that alloxan may exert its diabetogenic effect by acting on the beta cell membrane. These studies have been extended by determining the degree of lysis of washed red blood cells in alloxan solutions of varying tonicities.

Since alloxan rapidly decomposes at body temperature and pH, these studies were carried out at 0° and pH 7.0. It was shown that under these conditions the half-life of alloxan was over two hours. A stock solution of alloxan was neutralized to pH 7.0 by adding NaOH to give a final concentration of 0.308 *M* alloxan and 0.085 *M* sodium ion; the total tonicity was 0.393 *M*. The concentration of ionized alloxan is presumed to be 0.085 *M*; that of un-ionized alloxan would then be 0.223 *M*.

One hundred microliters of washed human red blood cells were added to 3 ml. of various dilutions of the stock alloxan solution. The degree of hemolysis was measured spectrophotometrically at 650 mμ. The results were compared with those obtained using various dilutions of NaCl and of phosphate buffer (Na_2HPO_4-KH_2PO_4, pH 7.0). With alloxan, 50% hemolysis was observed at a final tonicity of 0.118 *M*; the final alloxan concentration was 0.092 *M*. When NaCl was used 50% hemolysis was observed at a tonicity of 0.15 *M* (0.075 *M* NaCl). Phosphate buffer produced 50% hemolysis at a tonicity of 0.113 *M* (0.423 *M* phosphate). Thus, at equal tonicities alloxan and phosphate buffer produce a similar degree of hemolysis, suggesting that at 0° and pH 7.0 the red cell membrane is impermeable to alloxan.

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Secretory epithelium of the swim bladder in Fundulus. EUGENE COPELAND.

Preparations of the swimbladder epithelium from Fundulus were studied with the aid of the electron microscope. Earlier studies of Regaud preparations had revealed no obvious mitochondria under the light microscope. The electron microscope demonstrates fine filamentous mitochondria with tubular or bleb-like cristae. The nuclei show a characteristically deep infolding on one side. The free surface of the cells (gas facing) is smooth and uninterrupted. The basal surface (capillary facing) possesses deep infoldings similar to those described in the cells of the distal tubule of the kidney. Vesicles of varying density, frequently in rows, are associated with the folds. Within the double membranes of the folds, *i.e.*, exterior to the cell, can be seen density-free, vacuole-like spaces. These are assumed to contain oxygen mobilized by the bladder mechanism. Larger bubbles have been observed in the space between epithelial cells. Desmosomes connect the free ends of the cells at intervals but the impression is gained that gas escapes through areas or slots not so guarded. Patches of Golgi structure are seen. They have no obvious orientation in reference to the nucleus and the rest of the cell. Granules of varying size and density (lipid bodies?) are also seen. Correlation of their size and density with positional relationship to other structures has not been completed. All endoplasmic reticulum observed to date has been of the smooth variety. A very few "granular" strands were seen but they may well have been artifactual.

Oxygen toxicity among arthropods; in particular, modification of respiratory behavior and oxidative metabolism. F. EUGENE CORRIDEN.

Organisms across the entire taxonomic spectrum are known to be vulnerable to hyperoxia. Sensitivity is a variable of not only organism character but developmental stage and tissue type. Symptomatology has been rather well described. Commonly, the syndrome manifested includes an attenuation in respiration, suggesting an impairment of aerobic metabolism.

The provisional hypothesis adopted is that oxygen toxicity is mediated via an impedance of tricarboxylic acid cycle activity.

Insect forms frequently exhibit a depression in oxygen consumption subsequent to exposure to high tensions of oxygen (100%). Insects are known to possess a functional TCA cycle. Investigation has revealed that among pupal specimens of *Tenebrio*, pyruvic acid content (whole body) varies reciprocally with depression of oxygen consumption; the correlation is quite dramatic. Similarly, among pupal specimens of *Anagasta* and *Prodenia*, depression of oxygen consumption is accompanied by an accumulation of pyruvic acid and furthermore by a concurrent depletion of alpha-ketoglutaric acid. Pupal specimens of *Musca*, which did not exhibit any appreciable disparity in oxygen consumption, failed to show any modification in keto acid content. Pupal specimens of *Habrobracon*, in spite of a considerable retardation in oxygen consumption, maintained extreme stability in keto acid content. Extensions of experimental design to a study of *Uca pugnax* demonstrated toxicologic response to be paralleled by a rise in pyruvic acid content (muscle).

The tentative conclusion is that oxygen causes a block in the TCA cycle between the level of pyruvate and alpha-ketoglutarate. The speculation is that, specifically, the pyruvic oxidase system is the site of inhibition since it has a number of essential components known to be labile to oxygen *in vitro*.

Observations on the feeding activity of the isopod, Idothea baltica (Pallas). ARMANDO A. DE LA CRUZ.

Observations both in the laboratory and in the field were made on food preference, feeding behavior, rate and amount of feeding in the isopod, *Idothea baltica* (Pallas). These observations demonstrated that *I. baltica* is an omnivore that feeds readily on both the *Fucus* that harbors it and on living and dead animals. Values obtained from several experiments showed that on the average, an individual consumed about one-fifth of its body weight in half an hour when feeding on *Fucus*. When preying on a member of its own species, it consumed approximately half of its body weight in four hours.

It was demonstrated that the animals fed preferentially on the filiform processes on the surface of the *Fucus* thallus and on the younger and more tender portions of the plant. When feeding on other crustaceans, including individuals of their own species, they attacked living animals of up to one-half their own length, and dead or molting animals of almost their own size. They grasped their prey with all their pereiopods and usually feeding was initiated on the soft ventral parts. Eventually, the prey, including the exoskeleton, was totally consumed.

This work was undertaken as part of the student training program of the Marine Ecology Course and was partially supported by an ICA-NEC grant (Project No. 92-66-012-1-90197) and sponsored by the U. S. Office of Education, Department of Health, Education and Welfare.

Inhibitory action of a tissue extract on regeneration of Tubularia. AL. D. DINGLE.

Inhibition of regeneration of *Tubularia* has been obtained using supernatants of mature hydranth tissue breis at concentrations variously reported as ⅓ to ½ hydranth/ml. and 1⅓ to

2⅔ hydranths/ml. Wet weight determinations gave values ranging from 3 to 22 mg./hydranth, indicating that further quantitative studies should be based on hydranth weight rather than number. Supernatants, prepared by homegenizing washed mature hydranths in filtered sea water containing 125 μg./ml. streptomycin, and centrifuging the brei for 15 minutes at 10,300 *g*, were found to be inhibitory at concentrations between 15 and 30 mg./ml. The inhibitor was not species-specific, did not alter the pH of sea water, and its action could be simulated by equivalent concentrations of stem tissue (minus perisarc which contributes 20–40% of the wet weight).

When freshly cut stems were placed in the inhibitor, cellular protrusions usually capped the open ends, and further development stopped. Stems inhibited as long as 60 hours were capable of completely normal regeneration when returned to sea water. Application of the inhibitor to developing stems caused regression of any primordium already differentiated, and regenerated hydranths cytolyzed within 12 hours of treatment. Degeneration proceeded to the level of the neck in hydranths, and to the proximal end of the primordium in differentiating stems, then stopped—the stem did not cytolyze and remained capable of regeneration.

If the inhibitory supernatant is applied to a 2-mm. block of coenosarc left in the middle of a 10-mm. perisarc, the cells spread to fill the stem. This indicates that the extract does not act by stopping the migration of cells, and thereby preventing the build-up of a cell density critical for regeneration. It also demonstrates that the inhibitor does not act simply as a non-specific poison since the cells are able to undergo their normal movements.

Permeability studies on ground hog red blood cells. R. G. FAUST AND A. K. PARPART.

The relative rates of penetration of various monosaccharides and polyhydric alcohols into the ground hog red cell were studied by means of a photoelectric densimeter. Isosmotic concentrations (0.3 *M*) of these compounds were prepared in an isotonic NaCl-phosphate buffer which was adjusted to a desired pH. At pH 7 and 37° C., the rates of penetration to one-half diffusion equilibrium of the completely mutarotated sugars were as follows: for the pentoses; D-ribose 9 seconds, D-lyxose 16 seconds, D-xylose 22 seconds, L-xylose 24 seconds, D-arabinose 24 seconds, L-arabinose 29 seconds; for the hexoses: L-sorbose 33 seconds, D-galactose 57 seconds, D-fructose 125 seconds, and D-glucose 160 seconds. The disaccharide, sucrose, did not penetrate. There was no appreciable difference in the rates of penetration of the α- and β-isomers of D-glucose. Also at pH 7 and 37° C., the rates of penetration of the polyhydric alcohols are approximately linearly related to the number of carbon atoms in their molecule through 5 carbons. The rates were as follows: glycerol 1 second, i-erythritol 2.1 seconds, D-arabitol 3.5 seconds, L-arabitol 3.5 seconds, D-xylitol 3.5 seconds, ribitol 4.0 seconds, sorbitol 6 seconds, galactitol 9 seconds, and D-mannitol 11.5 seconds. The cyclic polyhydric alcohol, i-inositol, which contains 6 carbon atoms in its molecule, did not penetrate. The temperature coefficients (Q_{10}) which were calculated from the rates of penetration at 37°C. and 27° C. (at pH 7) were between 2.0 and 2.8 for all the sugars and between 1.2 and 1.6 for all the polyhydric alcohols that were tested. However, the influence of pH on the rates of penetration of the monosaccharides and the polyhydric alcohols was similar. Although pH variation affected their rates of penetration slightly, it was obvious that these compounds penetrated more slowly as the pH of their medium became more acid. Their rates of penetration were more rapid at pH 8 than at pH 6. This pH effect was reversible.

Comparison of the oxidation of C-1 and C-6 labeled glucose by islet tissue. JAMES B. FIELD AND ARNOLD LAZAROW.

The amounts of $C^{14}O_2$ produced from glucose labeled in the one and six positions, respectively, were measured; a ratio greater than one suggests the presence of the hexose monophosphate shunt. Islet, liver and heart were removed from toadfish and goosefish; these tissues were incubated with radioactive glucose at 22° C. for two hours. The carbon dioxide produced was collected in hyamine and the radioactivity was measured in a liquid scintillation counter.

The amounts of glucose oxidized to $C^{14}O_2$ by islet tissue were 3- to 10-fold greater than that observed for liver and heart. With islet tissue the amount of $C^{14}O_2$ produced from glucose labeled in the one position was several-fold greater than that produced from glucose labeled in the six position; this indicates the presence of the hexose monophosphate shunt in islet tissue.

Preliminary experiments were carried out in which varying amounts of either C-1 or C-6 labeled glucose were added to the islet tissue; the final glucose concentrations were 12.5, 25, 50, 100 and 200 mg. per 100 cc., respectively. The amount of C-6 labeled glucose oxidized by goosefish islet tissue increased progressively with increasing glucose concentration in the media; a maximal value was not reached at the highest glucose concentration (200 mg. per 100 cc.). By contrast, the amount of C-1 labeled glucose oxidized by islet tissue reached a maximum value at a glucose concentration between 25 and 50 mg. per 100 cc. At all glucose concentrations the ratio of CO_2 produced from C-1 vs. C-6 labeled glucose was greater than 2.0; at the lowest glucose levels, it was greater than 5.0. With liver the amounts of both C-1 and C-6 labeled glucose oxidized increased progressively with increasing glucose concentrations.

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Metachromatic granules in eggs of Hydroides. M. FILOSA.

Metachromatic granules in the cytoplasm of a number of marine eggs have been reported (Rebhun, Pasteels) to show specific movements during cleavage, in that they follow the centrioles. When unfertilized eggs of *Hydroides* are stained with toluidine blue (1:125,000) for five minutes, pink granules are found at random in the cortex of the egg. At no time during first or second cleavage of this egg do these metachromatic particles become associated with the mitotic spindle, but rather remain in the surface of the egg throughout. Eggs stained at first cleavage show the same localization of metachromatic granules as do eggs at the same stage that have been stained before fertilization. Eggs that were stained before fertilization were centrifuged at 8550 × g for both one and three minutes. When the germinal vesicle is intact, the metachromatic granules become localized near its centrifugal end. After the germinal vesicle has broken down, the location of the granules, up until first cleavage, differs, depending on the batch of eggs. In most cases the granules are moved internally and are located in the clear layer just below the lipid layer, with some granules scattered about in the clear layer. In some instances the granules form a layer in the yolk at the border of the clear layer.

Under oil immersion the metachromatic granules in these centrifuged eggs are seen to consist of two or three intensely stained small spheres on a light pink larger sphere. When unstained eggs were centrifuged at any stage between germinal vesicle breakdown and first cleavage, and then stained for five minutes, the metachromatic granules are always seen to lie in the clear layer just below the lipid cap. In these cases the particles are smaller and less intense in color than those of eggs that were stained before fertilization and centrifugation.

The influences of light and endocrines on the chromatophores of the mud shrimp, Upogebia affinis. MILTON FINGERMAN, R. NAGABHUSHANAM AND LORALEE PHILPOTT.

The anomuran *Upogebia affinis* possesses red chromatophores. Those on the telson and uropods can be readily observed and staged. Intact specimens on black and white backgrounds were placed under a series of light intensities ranging from 2 to 560 ft.c. No response to shade of backgound was observed. However, a response to intensity of incident illumination was apparent. The red pigment of specimens under an illumination of 2 ft.c. was maximally dispersed. With increased illumination (560 ft.c.) a slight decrease in the degree of pigment dispersion to stage 4 of the Hogben and Slome scale was noted in specimens on both backgrounds. To determine whether *Upogebia* displays a rhythm of color change, specimens were kept in darkness and in black pans under a series of light intensities ranging from 2 to 560 ft.c., and observed during the daytime and about midnight. No cycle was evident. However, the response to intensity of incident illumination was apparent at night as well as during the day. The red pigment was maximally dispersed in eyestalkless individuals and in intact specimens in darkness. The sinus gland of *Upogebia* lies on the dorsal surface of the supraesophageal ganglia rather than in the eyestalk. Extracts were prepared of the eyestalks, sinus glands, and supraesophageal ganglia, and injected into eyestalkless *Upogebia*. In all cases concentration of red pigment was observed. The least effect was produced by the eyestalk extracts. The responses to the extracts of sinus glands and supraesophageal ganglia were large and approximately equal.

This investigation was supported in part by Grant No. B-838 from the National Institutes of Health.

The responses of the melanophores of eyestalkless specimens of Sesarma reticulatum to illumination and endocrines. MILTON FINGERMAN, R. NAGABHUSHANAM AND LORALEE PHILPOTT.

Eyestalkless specimens of *Sesarma* were exposed for two hours to a series of incident light intensities ranging from 2 to 1110 ft.c. The degree of melanin dispersion in these eyestalkless crabs was a function of the incident light intensity. The melanin was maximally concentrated at 2 and 20 ft.c. With increased light intensity the melanin began to disperse. This pigment was almost maximally dispersed in specimens exposed to an illumination of 1110 ft.c. Melanophores on isolated legs showed a similar direct response to incident light, although the amplitude of the response was not as great as was observed when the legs were attached to the body. To determine the role of endocrines in pigment migration in *Sesarma,* extracts were prepared of the sinus gland, optic ganglia, supraesophageal ganglia, circumesophageal connectives, and thoracic nerve cord. These extracts were injected into eyestalkless *Sesarma* whose melanin was maximally concentrated, and into specimens maintained under a high intensity of illumination so that the melanin was in a state intermediate between the fully dispersed and fully concentrated conditions. Each extract dispersed melanin. Extracts of sinus glands produced the least dispersion. In the case of the sinus glands, after the dispersing action of the extract, pigment concentration was noticed in the specimens whose pigment had been in an intermediate state at the time of injection of the extracts. Although the amount of melanin was not large, it was statistically highly significant.

This investigation was supported in part by Grant No. B-838 from the National Institutes of Health.

Responses of the melanophores of the grapsoid crab Sesarma reticulatum to light and temperature. MILTON FINGERMAN, R. NAGABHUSHANAM AND LORALEE PHILPOTT.

Sesarma exhibits a cycle of color change whose frequency closely approximates 24 hours. Crabs were placed in darkness and observed daily at 8–8:30 A.M., noon–1 P.M., 5 P.M., and 12 P.M. for one week. The pigment was less dispersed at midnight than at other times of day or night. Crabs were then tested to determine whether differences in incident light intensity and in shade of background would have an influence on the degree of melanin dispersion. During the daytime the melanin was maximally dispersed in animals kept in black pans under a series of light intensities from 2 to 1110 ft.c. At 2 ft.c. the melanin of animals in white pans was in an intermediate stage of dispersion. With increase in intensity of incident illumination a smoothly graded response was noted; the melanin became maximally dispersed in specimens in white pans under an illumination of 1110 ft.c. The responses to black and to white backgrounds and to incident light intensities between 2 and 1110 ft.c. were then determined between 11 P.M. and 3 A.M. The melanin of crabs in white pans was more concentrated than that of crabs in black pans at all light intensities. A response to intensity of incident illumination was also apparent; the melanin was more dispersed in crabs under the high intensities than under the lower ones, whether the crabs were in black or in white containers. However, at night the melanin did not disperse maximally in crabs on a black or a white background under any of the light intensities used. Responses to temperature were determined during the daytime with crabs in white pans under an illumination of 2 ft.c. At 3° C. the melanin was more dispersed than at 36° C.

This investigation was supported in part by Grant No. B-838 from the National Institutes of Health.

Effects of Blepharisma pigment on marine invertebrate development. B. A. FONG AND H. I. HIRSHFIELD.

The fresh-water ciliate, *Blepharisma undulans,* contains a red pigment that is photolytic and cytotoxic to a variety of cells and organisms. Initial studies were made on the effects of the pigment on gametes and developmental stages of the marine annelid, *Chaetopterus pergamentaceus,* and the sea urchin, *Arbacia punctulata.* These two species were selected since they are members of two major superphyla. Accordingly, differential responses to the pigment of these two species may have possible evolutionary significance.

The alcohol-extracted pigment was dried and a 1 mg./ml. concentration was prepared. This concentration and serial dilutions in sea water of 0.1, 0.01 and 0.001 mg./ml. were used.

The effects of the pigment in an intense light field and in the dark were noted on the gametes and developmental stages of the two species. A significant decrease in egg fertility was obtained, whereas the inseminated eggs showed less developmental delay and cytolysis. Exposure to light enhanced the cytolytic damage considerably.

Chaetopterus eggs exposed to the maximal concentration of pigment for 90 minutes, prior to insemination with untreated sperm, showed large numbers of abnormal and unhatched blastulae. In striking contrast, *Arbacia* showed abnormal fertilization membranes and 99% mortality. Those eggs of *Arbacia* that survived showed either abnormal cleavage patterns or developed normally through blastulation and hatching. Large numbers of abnormal larvae of both species were seen in the pigment-exposed groups.

Light and dark controls were kept for each series.

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Transplantation of distal limb tissues to upper arm stumps in adult Triturus viridescens. RICHARD W. GLADE.

Pertaining to the problem of the establishment of pattern during limb regeneration, the following experimental series were performed: (1) implantation of three ulnae per stump (9 cases); (2) implantation of the phalanges of three digits per stump (12 cases); and (3) transplantation of a sheath of forearm skin to encircle the stump (10 cases). The bone transplants carried some adherent connective tissue and muscle. The skin grafts, however, rarely included muscle, and then only as an occasional fiber. Two control series involved the implantation of the distal halves of two humeri (15 cases), and the implantation of the proximal halves of two mandibles (11 cases) per stump. In all series, mock operations were performed on the opposite forelimbs, which served as controls.

Regeneration of the experimental stump was slightly delayed, beginning with the blastema or cone stage in the ulna, phalanges, and mandible series (6/9, 8/12 and 11/11 cases, respectively) as compared with its control. Delay occurred in 7/15 cases in the series in which humeri were implanted. In all of the remaining animals, the experimentals and controls regenerated at equal rates. The forearm skin series differed from the above in that in only 2/10 cases were the experimental regenerates delayed, while in 3/10 cases the controls were delayed. In 5/10 of the cases regeneration of the two limbs occurred at an equal rate.

Concerning the morphology of the regenerates, a variable degree of shortening in the length of the forearm, without significant inhibition of the hand, was observed in the ulna, phalanges and skin series. In no animal was the control forearm regenerate shorter than the experimental. An histological examination of the regenerates is being carried out.

This work was done with the technical assistance of Miss Nancy J. Scott.

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Movements of ^{24}Na and ^{42}K in the squid retina. W. A. HAGINS AND R. G. ADAMS.

Exchange of ^{24}Na and ^{42}K with sodium and potassium in the isolated squid retina has been measured. The retinas were isolated in darkness under an infra-red image converter and mounted in a thin chamber between two rapidly flowing layers of artificial sea water. Isotope solutions were flowed past the retina and then washed away with non-radioactive ASW and the activity remaining in the tissue monitored with a counter placed against the chamber. The responses of the retina to light were measured with electrodes fixed in the chamber. ^{24}Na moved into 30% of the retinal water within one minute and could be washed out with similar speed. Long exposure to ^{24}Na revealed an additional slow uptake into a further 10% of the retinal water in two hours. This latter compartment lost its ^{24}Na slowly with an exponential time course whose half-time was 30 minutes at 10° C. ^{42}K uptake by the retina was slow and roughly exponential, with 70% of the intracellular K^+ exchanged in 3 hours. ^{42}K outflux was also slow and precisely exponential. It was not affected by raising the external K^+ from 10 to 30 mM even though the retinal photocurrent was reduced 30%. Similarly, 2 mM procaine left the outflux unaffected while increasing the photocurrent by 60%. In procaine-ASW, light which caused a total flow of 0.02 micromole of ions per cm.^{2} of retina produced no change in the ^{42}K outflux, though an increased total outflux of this size would have been just detectable. In the absence of procaine, light did produce a small increase in ^{42}K outflux lasting for 10 minutes after cessation of illumination. Light adaptation which reduced the retinal sensitivity 30-fold left the fluxes unaltered and the K content of the cells essentially undisturbed. Since the exposure used isomerized less than 1% of the retinal photopigment, light adaptation must involve changes other than simple destruction of rhodopsin or gross changes in the Na and K content of the receptors.

Light-induced current from the receptors of the squid retina. W. A. Hagins, R. G. Adams and H. G. Wagner.

Isolated, dark-adapted squid retinas at 10° C. bathed in flowing oxygenated artificial sea water (ASW) maintain their sensitivity to light for many hours. Characteristically, the retina responds to the onset of illumination with a transretinal voltage rising sigmoidally from nearly zero to a steady level. The value reached is directly proportional to light intensity between 10^6 and 10^{11} photons cm.$^{-2}$ sec.$^{-1}$ (500 mμ). When the light is turned off the voltage returns rapidly to zero. The rise and fall of the voltage show a latency of about 80 msec. and a transition time of about 100 msec.; the exact values depend on temperature. Since the electrical impedance of the retina is essentially an ohmic resistance of 20–30 ohm cm. between 5 and 5000 sec.$^{-1}$, a light-induced transretinal voltage implies the flow of current within the retina. With the two surfaces of the retina short-circuited through a feedback amplifier, measurement of the current shows it is of the order of 10^5 electronic charges per incident photon (500 mμ). The origin of the photocurrent is attributed to the photoreceptors since (1) these cells are the bulk of the retina and the principal nervous elements, and (2) the action spectrum of the photovoltage closely resembles the absorption spectrum of squid rhodopsin, and (3) the amplitude of the photovoltage varies with depth as recorded by micro-electrode, consistent with the presence of a positive current arising in the region of the receptor cell bodies, flowing longitudinally between them and ending near their photo-pigment-bearing ends. The electro-chemical nature of the current source has been studied by ionic substitution in the solutions bathing the retina. Replacement of Na^+ by choline$^+$ reversibly reduces the photocurrent by 30%, as does increasing the external [K^+]. Conversely, removal of K^+ from ASW increased the current slightly while 2.0 mM procaine increased it 50%. It is thus suggested that the photocurrent depends in some way on the membrane potential of the receptors but not on the presence of electrical excitability in the receptor axons.

The effect of polyvinylpyrrolidone on volume of the isolated rat lens. Clifford V. Harding.

When isolated rat lenses are maintained in Ringer-Locke solution at low temperature, they slowly increase in volume and lose transparency. It has been suggested that the increase in volume is due to the colloid osmotic pressure of the lens, which would become effective following the gradual inactivation of ion transport mechanisms under the *in vitro* conditions employed. Other explanations for the swelling are possible. This suggests, however, that lenses maintained in a solution of proper colloid osmotic pressure would not undergo an increase in volume. The earlier work of Pau with both lens and cornea demonstrated this to be the case. In the present studies, rat lenses were maintained at approximately 5° C. in Ringer-

Locke containing various concentrations of polyvinylpyrrolidone (PVP), a synthetic polymer which can be obtained at different molecular weights. The two molecular weights used were approximately 40,000 (K-30) and 360,000 (K-90). It was found possible to control lens volume by adjusting the concentration of PVP in the medium. For example, in low concentrations of K-30, the lens swells; in high concentrations, it shrinks, and at 15%, approximately normal volume is maintained and transparency is not lost over long periods of time (48 hours and longer). Regardless of the mechanism whereby PVP controls the volume of the lens, it is suggestive that a proper adjustment of the colloid osmotic pressure of the medium can prevent the swelling which normally follows lens isolation. This could conceivably avoid damage to cells and fibers, which might accompany lens swelling, and thus maintain the lens in a viable state *in vitro* for longer periods of time.

It is planned to check this by measuring the extent of cellular proliferation in the lens epithelium, a factor which is apparently a sensitive index of the viability of the lens.

Colloid osmotic pressure might also be the effective characteristic of solutions of PVP which have been shown to prolong the time that rabbit corneas can be maintained *in vitro* without losing their capacity for transplantation (La Tessa).

Thymidine incorporation in epithelium of fish lens maintained in vitro. CLIFFORD V. HARDING AND B. D. SRINIVASAN.

Tritium has been localized autoradiographically in whole-mount preparations of lens epithelium, a single layer of cells, from the rabbit and rat. The use of whole-mounts for this purpose makes possible a comparison of the extent of incorporation of tritium-labelled compounds in one portion of the epithelium with any other portion, in one preparation. This procedure has proven useful in studies on the incorporation of tritium-labelled thymidine in normal and injured epithelium.

It was thought of interest to extend this study to the lenses of cold-blooded animals. These would have the advantage of enabling a study of temperature effects in the intact animal under physiological conditions; and, they would also, perhaps, furnish a more favorable material for *in vitro* studies on cell division. Results of the present investigation have shown that the whole-mount method can be applied to the lens epithelium of dogfish, tautog, and sea bass. Freshly isolated dogfish lenses were incubated for two hours in a solution of elasmobranch Ringer (made up in glass-distilled water, pH approximately 7.5) plus tritium-labelled thymidine at 2.5 to 5 μc/ml. (Spec. act. 1.9 curies/mM), after which they were fixed in 3 parts absolute alcohol: 1 part glacial acetic acid. Whole-mounts were then prepared for autoradiography. The autoradiograms were exposed for 7 to 17 days before photographic development. The results show incorporation by many of the epithelial cells. The cells showing incorporation are relatively more scattered than in the rabbit lens, where the germinative zone is relatively localized.

Experiments were also carried out in which dogfish lenses were incubated in elasmobranch Ringer (with dextrose) for various periods of time before exposure to thymidine. Lenses maintained in elasmobranch Ringer at 21° C., for at least up to 22 hours before a two-hour exposure to tritium-labelled thymidine, showed incorporation of thymidine. Similar results were obtained with the epithelium of tautog lenses, maintained in teleost Ringer at 16° C. for 22 hours. The results suggest the possible use of this tissue for *in vitro* studies on cell division.

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Stimulation of Arbacia sperm respiration by egg substances. RALPH R. HATHAWAY.

Arbacia fertilizin is generally thought to depress the respiration of specific sperm. In a reinvestigation of this question, *Arbacia* sperm respiration and motility were markedly increased by a substance which freely diffuses from eggs. This *Arbacia* sperm-activating substance was prepared as the sea water supernatant of jellyless eggs. Such a sperm-activating preparation has no sperm-agglutinating properties, is diffusible in dialysis, soluble in alcohol, heat-stable, and non-volatile. The activator is evidently a substance distinct from fertilizin.

When *Arbacia* sperm activator is mixed with an excess of specific sperm in glycyl-glycine buffered sea water, there is a four- to 20-fold increase in respiration rate. The high rate of O_2 consumption rapidly decreases as a power function of time. When the activator is in excess, the elevated respiration rate slowly decreases as a linear function of time. The pH is essentially constant throughout. When activator-sperm mixtures are centrifuged after one-half hour, the supernatants no longer contain activating properties. Conversely, sperm washed from the activator after one hour do not respond to fresh activator preparation. These facts suggest a mutual exhaustion of the activator and a sperm substance essential for sperm response to the activator.

Sperm activator occurs in *Arbacia* eggs from Woods Hole, Mass., and Alligator Point, Florida. Sperm activators were also found in *Lytechinus variegatus* and *Mellita quinquiesperforata* from Alligator Point. *Asterias forbesi* eggs from Woods Hole yielded no sperm activator. In specificity studies, *Arbacia* activator failed to stimulate sperm of *Lytechinus, Mellita* and *Asterias. Lytechinus* sperm activator did not affect *Arbacia* sperm, but *Mellita* activator caused a moderate increase in *Arbacia* sperm respiration.

Sperm of *Arbacia, Lytechinus* and *Melita* have invariably displayed an increased oxygen consumption in the presence of homologous fertilizin. This response may be due to the presence in fertilizin preparations of small quantities of active sperm activator substance(s).

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Egg jelly dispersal by Arbacia sperm extracts. RALPH R. HATHAWAY, LEONARD WARREN AND JOEL G. FLAKS.

Various substances, some containing sialic acid, are released by *Arbacia* sperm when they are treated with either *Arbacia* fertilizin or *ca.* 10^{-4} *M* sodium lauryl sulphate (see separate abstract). These sperm extracts affect *Arbacia* eggs by causing the disappearance of the egg jellies in 15 to 60 minutes at room temperature. Eggs which have lost their jelly coats are easily recognized by their close contact with each other. In addition, when *Arbacia* eggs are exposed to *Asterias* sperm, the egg jellies become impregnated with trapped non-fertilizing sperm. Subsequent exposure of these eggs to *Arbacia* sperm extracts results in the disappearance of the jelly coat and the release of the starfish sperm. This observation suggests that the disappearance of the egg jelly is caused by a true jelly dispersal rather than by a precipitation and contraction of the jelly, as is sometimes reported when other sperm extracts, *e.g.,* antifertilizin, are mixed with eggs. Furthermore, in preliminary tests supernatants from eggs treated with the sperm extracts contained more fucose than supernatants of control eggs. Since fucose is a major constituent of the egg jelly, this result indicates solubilization of the egg jelly coat.

The jelly "dispersing" activity is destroyed by heat (80° C. for 5 minutes), and acid (pH 3.0 for 5 minutes), and is inactive at 0°. These properties distinguish this agent from antifertilizin, and suggest an enzymatic activity.

The sodium lauryl sulfate extracts of sperm contain antifertilizin which precipitates a membrane around the jelly coat. Observation of the jelly "dispersing" activity of these preparations can be made only after the antifertilizin is removed. The jelly precipitating activity can usually be removed by centrifugation at 33,000 *g* for 35 minutes. The pellet contains the jelly precipitating activity and all of the sialic acid which was extracted from the sperm by the sodium lauryl sulfate. The 33,000 *g* supernatant contains a strong jelly coat "dispersing" activity.

Aided by National Institutes of Health (RG-6234), to Dr. Charles B. Metz.

The pseudocilia of Tetraspora. WALTER R. HERNDON AND DELBERT E. PHILPOTT.

The immobile, paired, pseudocilia of the vegetative cells of the chlorophycean alga *Tetraspora lubrica* Roth strongly resemble the flagella of its motile cells when examined microscopically, especially with lower magnifications. However, with oil immersion and phase contrast, it is clear that these protoplasmic strands have a structure different from that of typical flagella; they are comprised of an axial portion surrounded by a relatively broad sheath. The axial portion is of greater optical density than the sheath and is beaded; the sheath stains readily with methylene blue. Pseudocilia of colonies fixed in 1% osmic acid or 5% potassium permanganate, embedded in methacrylate and sectioned show the same structure as seen in the living material when examined with the light microscope, except there is in some a slight shrinkage of the sheath and in these the axial portion may be somewhat coiled. In sections thus prepared and examined with the electron microscope, the sheath and axis are clearly distinguished. The sheath is about one micron in diameter and relatively uniform along its length. The axial portion appears to be basically tubular but has numerous constrictions which delimit spherical and elongate segments of varying sizes (mostly 0.2–0.4 micron in diameter). At its insertion, the axial portion appears to be an extension of the anterior cytoplasm peripherally disposed about a vacuolate interior. The beaded structure of the pseudocilia was found in colonies of different ages, in collections from different localities, and in a different species of the genus. The position of the pseudocilia of vegetative cells and some features of their morphology suggest that they might be produced by a process which is a modification of that producing the typical flagellar structure of motile cells also examined with the electron microscope, for comparative purposes, in this study.

The stage at fertilization of the egg of Fundulus heteroclitus. CHARLES W. HUVER.

According to Costello, Davidson, Eggers, Fox and Henley (1957) in *Methods for Obtaining and Handling Marine Eggs and Embryos*, the stage at which the egg of *Fundulus heteroclitus* is fertilized has not been determined. This is due to the fact that the polar bodies of this popular embryological material have not been previously described.

While examining time-lapse movies of fertilization and cytoplasmic streaming during blastodisc formation, both polar bodies were observed, apparently for the first time. The polocytes have now been observed in a total of 15 eggs. They project into the perivitelline space from the rim of the funnel-shaped mouth of the micropyle. The first and second polar bodies are of equal size (0.04 mm.-diameter). They are in close contact with each other and are incompletely elevated from the egg surface.

The reasons why students of early *Fundulus* development have previously failed to observe the polar bodies are: (1) the mature egg is very difficult material for proper fixation and sectioning, (2) the extreme transparency of the polocytes, (3) the relatively small size of the polar bodies in relation to the size of the egg, (4) the resemblance of the polar bodies in size and in shape to cortical alveoli, and (5) the masking effect of the thick chorion which encloses the egg.

The first polar body becomes clearly visible upon the elevation of the chorion at approximately three minutes after insemination. The time of emergence of the first polar body is unknown. However, it appears to be present in freshly stripped unactivated eggs where it is closely compressed to the egg surface. The actual emergence of the second polar body can be seen in fertilized eggs. It arises next to its predecessor in a smooth but quick movement at 4.5–5.0 minutes after insemination. The presence of the first polocyte in the ripe unfertilized egg, and the rapid formation of the second after sperm entrance, indicate that the egg is a secondary oocyte when fertilized. The emergence of the second polar body so soon after insemination suggests that the second maturation division proceeds to an advanced stage before it reaches the pre-fertilization arrest.

N.I.H. Predoctoral Fellow.

Effects of temperature on charge transfer through a receptor membrane. NOBUSADA ISHIKO AND WERNER R. LOEWENSTEIN.

Mechanical stimulation of the nerve ending of Pacinian corpuscles produces transfer of charges through its receptor membrane. The energy requirements for the transfer are markedly influenced by temperature. For example, the mechanical stimulus strength necessary to produce a given generator potential at 25° C. may be reduced to one-third at 35° C. A temperature change alone, however, elicits no detectable transfer.

The main effect of a temperature change is to vary the rate of rise and the amplitude of the mechanically elicited generator potential. Both increase linearly with temperature, with

Q_{10} of 2.5 and 2.0, respectively (15° C.–35° C.). The primary event here appears to be the effect on the rate: the active phase is shorter than the rise time of the generator potential and, hence, the total charge transferred increases as the transfer rate augments with temperature. The rate of fall of the generator potential is not appreciably affected by temperature.

The first Ranvier node, the site of nerve impulse initiation, behaves quite differently. The amplitude of the nodal action potential remains practically unchanged between 20° and 40° C., and its duration increases considerably with temperature (Q_{10}, 3–4). The nodal threshold varies inversely with temperature; impulse initiation fails completely below 12° C., although the receptor membrane still produces generator potentials.

A simple model for receptor excitation is that of ions diffusing simply along their gradients through mechanically stretched pores of the receptor membrane. This model is now no longer tenable without modification. The high activation energy of the rate-limiting process in excitation, 16,300 cal./mole (as derived from the temperature dependence of the rate of rise of the generator potential), forces at least one additional element upon this or any other model, namely an energy barrier for charge transfer. To surmount the barrier, energy may be supplied directly by heat transfer, or indirectly by a chemical reaction.

Stimulation of conidia formation at the growing tips of Neurospora crassa by x-irradiation. J. Keosian and B. P. Sonnenblick.

N. crassa grows with a sharp frontier in an agar minimal medium in growth tubes. When this frontier is x-irradiated (9000 r at 182 kvp) the following are noted:

1. Formation of a dense band of conidia, about 5 mm. wide, at the site of the original irradiated frontier, visible in 6–8 hours. This band persists as to location, width and relative density, even after the tube is overgrown with conidia.
2. The conidial band forms when the frontier alone is irradiated and the rest of the growth is lead-shielded, but the band does not form when the frontier is shielded and the remainder exposed. This suggests that the radiation influence is limited to the growing tips.
3. Age appears to have no significant effect on the formation of the conidial band since irradiation of 24-, 48- and 72-hour cultures produced comparable results.
4. The threshold for the formation of the band is between 1000 r and 2000 r, resulting in a definite but sparse band. With increasing dose the band becomes denser with conidia. Experiments performed with a previous machine (85 kvp) indicated an optimum effect at about 9000 r.
5. Retardation of growth rate for the first six hours, followed by an acceleration of growth rate over normal. This increase is small but is consistently found in the irradiated cultures.
6. Conidial band formation at the original frontier, with visibly different characteristics from that induced by radiation, occurs after exposure for given periods of time at certain temperatures (10° C. and 42° C.).

Histochemical and biochemical analyses are projected in an attempt to ascertain whether the localized conidial band formation described above is a generalized stress response to an unusual environment, or whether there are differences in the response to radiation *per se* and to other agents.

Observations on the submicroscopic cytology of the epithelial cells of the cardia of a dipteran insect, Hypoderma bovis. Richard G. Kessel.

The cardia of dipterous insects is the most anterior portion of the midgut. It is known to be highly active in secretion and involved in the production of the peritrophic membrane. The lumen surface of the epithelial cells possesses a multitude of microvilli, often arranged in hexagonal array. Numerous blebs of the apical portion of the plasma membrane can be observed. A budding or blebbing of the distal portion of the microvilli is also apparent, but on a smaller scale. The most abundant cellular organelle is the endoplasmic reticulum. In the basal portion of the cells, the reticulum consists of numerous, parallel, interbranching, rough-surfaced lamellae. In the central portion of the cell, the lamellae of the endoplasmic reticulum are observed to be greatly expanded at their end region (forming ergastoplasmic sacs) or along their length. In the most apical portion of the cell, numerous smooth- and rough-surfaced vesicles are characteristically present. The latter may also be observed within the large blebs of the apical plasma membrane. It appears that the secretory vesicles have their origin from the endoplasmic reticulum. Mitochondria and parallel, smooth-surfaced lamellae of the Golgi apparatus are also present, but both are extremely small. The Golgi material was never observed to be conspicuous. The cells abut upon a basement membrane approximately 0.75 micron thick. The nuclear membrane is porous and a prominent nucleolus is present. Observations at various stages in the formation of the peritrophic membrane suggest that the microvilli contribute, at least in part, to its formation. (Supported by a grant, RG-6942, from the National Institutes of Health, U.S.P.H.S.

An electron microscope study of the mitochondria-rich "chloride cells" from the gill filaments of fresh water- and sea water-adapted Fundulus heteroclitus. RICHARD G. KESSEL AND H. W. BEAMS.

The ultrastructure of the gill filaments of *Fundulus heteroclitus* was studied with special reference to the mitochondria-rich chloride cells which were earlier described with the light microscope by Copeland (1948, 1950). Filaments of animals from sea water were examined, as well as those from animals adapted to fresh water for twelve hours.

In sea water-adapted animals, the chloride cells appear very dense, due to the numerous, filamentous mitochondria. The cristae of the mitochondria are mainly oriented parallel to its long axis. Located among the mitochondria and closely associated with them is a prominent, tubular network of an agranular type. This structure is similar to a specialized form of endoplasmic reticulum described in the pseudobranch gland (Copeland, 1959). The nucleus is characterized by two areas of different electron densities, each of which appears lobated.

In fresh water-adapted animals, the chloride cells do not appear to be as densely populated with mitochondria. In addition, the mitochondria appear shorter and thicker than those in sea water and the cristae in many cases are oriented transversely. The network of smooth-surfaced, branching tubules (thought to represent a form of the endoplasmic reticulum) appears more abundant, but smaller in size, than is the case in sea water. A continuity of the tubular endoplasmic reticulum and the plasma membrane of the cells is observed under both sea water and fresh water conditions. A small amount of rough-surfaced lamellae of the endoplasmic reticulum is also present. The nucleus appears homogeneous, is of moderate electron density and often contains a nucleolus. (Supported by grants from the National Institutes of Health, U.S.P.H.S.)

The effect of waves in influencing the composition of a flora of attached sea-weeds. JOHN M. KINGSBURY.

The granite jetty projecting across the harbor entrance at West Falmouth, Mass., lies parallel to the general shore outline of Buzzards Bay. Its western, bay exposure (with 69% of total wind over open water fetch of 6¼ miles or more) receives constant wash of waves or swells, usually producing spray. Its eastern, harbor side (maximum fetch less than ½ mile, wind exposure about 2%) rarely experiences waves and never has significant spray. Tidal effects, water temperature, light, currents, and rock substrate were observed or measured and found identical or nearly so at the six collecting stations. Stations were paired, leeward and windward members opposite, the first pair 10 feet from the projecting end, second and third pairs each 12 feet further to landward. At each station all manually removable vegetation was collected from 6-inch quadrants forming a vertical transect from highest growth to jetty bottom.

A *Calothrix* zone, above lowest high water, was present only to windward. A 3-foot (vertical) zone of *Fucus vesiculosus*, present on both sides, was ½ to 1 foot lower on the leeward side. Its lower boundary leeward corresponded with extreme low tide level; windward it was bounded by mean low and lowest high tide levels. The leeward side showed fewer germlings, presence of bladders and larger plants in comparison with windward. A zone, between highest and lowest low tide levels, dominated by *Enteromorpha linza*, occurred only on the windward side. Of 35 species found on windward, 8 were not found on leeward; similarly, 2 of 29 leeward species were not found on windward. *Chondrus crispus*, the principal non-epiphytic member below the *Fucus* and *Enteromorpha* zones, was displaced upwards 6 to

9 inches on the leeward side. On the leeward side the vertical level equivalent to the windward *Enteromorpha* zone was occupied chiefly by the downward *Fucus* and upward *Chondrus* displacements.

Centrifugal influence on the electroretinogram of the frog. STEPHEN T. KITAI.

The existence of centrifugal fibers in the optic nerve was investigated by studying the effects of Nembutal on the electroretinogram b-wave responses of frogs under the following conditions: (1) spinal pithing, (2) spinal pithing and unilateral optic nerve sectioning before the injection of the Nembutal, and (3) section of the optic nerve after the Nembutal effect on the electroretinogram responses appeared.

The electroretinogram responses were recorded from the cornea of frogs, using sodium chloride-plated silver electrodes. The signals from the electrodes were fed into Grass P6 d.c. pre-amplifier and finally recorded by an oscilloscope. The electroretinogram records were taken before and after the Nembutal injection under spinal pithed condition, and under spinal pithed unilateral optic nerve-sectioned condition.

Changes in the magnitude of the electroretinogram responses due to extraneous variables, such as changes in the retinal area stimulated or in the amount of stimulus light, were controlled. Optic nerve sectioning did not damage the retinal artery. The electroretinogram responses from the unoperated contralateral eye were taken as a control.

The effect of Nembutal on frogs under spinal pithed condition was to decrease the electroretinogram responses. The Nembutal injection failed to decrease the electroretinogram responses when the optic nerve was sectioned before the injection. The decreased electroretinogram responses by Nembutal injection were eliminated when the optic nerve was sectioned.

Sectioning of the optic nerve abolished the effects of Nembutal on the electroretinogram b-wave responses. This is interpreted as an evidence for the existence of the centrifugal fibers in the optic nerve, since the higher center upon which the drug acts no longer connected with the retina.

Krebs cycle dehydrogenase systems in eggs of Asterias as measured with a tetrazolium salt. EVELYN KIVY-ROSENBERG, FRANCES RAY AND HELENE ELEFANT.

The quantitative study of substrate-dependent dehydrogenase activity has been in progress for several years (Kivy-Rosenberg, Cascarano and Merson, 1959; Biol. Bull. 117). Work was continued on *Asterias* uninseminated and inseminated eggs. An extensive series of substrates was utilized, including those which require DPN or TPN as cofactors and succinate which requires none. Included in this series were four which are involved in the Krebs cycle: alpha-ketoglutarate, and malate, each with DPN, isocitrate with TPN, and succinate. The tetrazolium salt used as a hydrogen acceptor was 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT).

Homogenates of uninseminated and inseminated eggs of the same female (of a large series of starfish) were incubated aerobically for one hour at 37.5° C. in media containing INT and substrate or appropriate controls. Formazan was extracted and the quantity of reduced tetrazolium measured photocolorimetrically. The substrate-dependent dehydrogenase activity was expressed as micrograms of formazan per milligram of protein.

Of the fifteen hydrogenase systems measured, three in the Krebs cycle are among the most active: alpha-ketoglutarate ranking first; isocitrate, second; malate, third, in practically all cases. Occasionally ranks of alpha-ketoglutarate and isocitrate were interchanged, and malate ranked fourth. The activity of the other member of the cycle, *i.e.*, succinic dehydrogenase, was among the lowest of the entire series. The ranks mentioned were similar for both uninseminated and inseminated eggs.

Insemination resulted, in general, in a rise of dehydrogenase activity in homogenates (as we had reported earlier also for *Spisula*). In a few cases the reverse was true. On the whole, the activity of the Krebs cycle dehydrogenases followed the general trend: *i.e.*, a rise (when there was a general rise with insemination), and a fall (when the reverse was true). This was true for the one which showed relatively low activity as well as the three highly active dehydrogenases.

Further consideration and study will be given to the surprising relationship between the low activity of succinic and high malic dehydrogenase systems.

The iron requirement of some marine plankton algae. DANA D. KRAMER AND JOHN H. RYTHER.

The iron requirement of some marine phytoplankton was investigated by determining final populations attained in media containing different concentrations of Fe-EDTA. The organisms studied were three oceanic species (*Coccolithus huxleyi, Chaetoceros* sp. and an unidentified centric diatom "13-1") and four inshore species (*Skeletonema costatum, Isochrysis galbana, Amphidinum carteri* and an unidentified flagellate "3C"), all maintained in pure culture at the Woods Hole Oceanographic Institution.

The algae were grown in offshore ocean water enriched with all essential nutrients except iron, until further growth was obviously iron-limited. Iron-deficient cells were then inoculated into media enriched respectively with 0, 2.6, 6.5, 13, 26, 65 and 130 μg. Fe/L. These cultures were incubated (1000 foot candles, 20° C.) until maximum growth was attained (*ca.* 7 days), after which population density was determined by cell counts.

Coccolithus could not be made iron-deficient. After several transfers, more growth occurred in media without than with iron added. All other species became iron-deficient after 2–3 transfers, with no growth in control cultures containing no added iron. The two other offshore species (*Chaetoceros* sp. and "13-1") reached maximum densities at 26 and 2.6 μg. Fe/L, respectively; growth was depressed at higher levels. Of the inshore species, *Isochrysis* and *Amphidinum* reached maximum growth at 65 μg. Fe/L; populations of *Skeletonema* and "3C" were still increasing at the highest iron concentration employed.

Skeletonema, an unbiquitous shallow-water diatom never found in the open sea, does not grow at iron concentrations supporting maximum growth of the oceanic species *Coccolithus* and "13-1." It is suggested that the higher level of iron in coastal waters associated with land drainage may influence or control the distribution of this and other neritic species. This work was carried out as part of the student training program of the Marine Ecology Course.

Effect of nicotinamide on pyridine nucleotide levels and cleavage rate of eggs of Spisula solidissima. STEPHEN M. KRANE AND ROBERT K. CRANE.

It was previously demonstrated in this laboratory that the major change in the concentration of pyridine nucleotides in early cleavage stages of marine eggs was a net increase in triphosphopyridine nucleotide in its reduced form (TPNH). In the present study, eggs of *Spisula solidissima* were incubated with nicotinamide in an attempt to modify the levels of pyridine nucleotides as well as the rate of cleavage. Pyridine nucleotides were measured fluorimetrically. The concentration of diphosphopyridine nucleotide (DPN) in unfertilized eggs incubated in nicotinamide (1×10^{-2} *M*) in sea water increased linearly from 210 millimicromoles/ml. cells to 535 millimicromoles/ml. cells in three hours. With lower concentrations of nicotinamide the increase was more striking (1010 millimicromoles/ml. at 1×10^{-4} *M* nicotinamide; 781 millimicromoles/ml. at 1×10^{-5} *M* nicotinamide). In eggs transferred to nicotinamide (1×10^{-2} *M*) in sea water 40–60 minutes after fertilization, the DPN levels were doubled, compared to controls three hours later, and cell division completely but reversibly blocked. Cleavage was blocked in concentrations as low as 5×10^{-4} *M* but only delayed at 1×10^{-4} *M*. Unfertilized eggs incubated three hours in nicotinamide (1×10^{-4} *M*) showed no change in TPNH levels compared to controls (5 versus 4 millimicromoles/ml.) despite marked increase in DPN (820 versus 194 millimicromoles/ml.). In fertilized eggs in nicotinamide, TPNH was 72 millimicromoles/ml. versus 34 millimicromoles/ml. in controls, whereas DPN was 1280 millimicromoles/ml. versus 185 millimicromoles/ml. in controls.

Effects of nicotinamide in *Spisula* eggs were therefore as follows: (1) Reversible inhibition of cell division; (2) Increase in levels of DPN in fertilized and unfertilized eggs; (3) Increase in TPNH *only* if cell division had proceeded.

Mechanisms of nerve impulse generation in a Lorenzinian ampulla. WERNER R. LOEWENSTEIN.

Single isolated ampullae of dogfish (*Mustelus canis*) were enclosed in a pressure chamber. The ampulla tubule was cannulated and connected to a pump. Thus, the internal and/or external pressure of the ampulla could be varied over a wide range. Single axons were dissected up to their point of emergence from the ampulla, and electrical activity was led off that point. Generator and action potentials were thus recordable.

At normal atmospheric pressure only a few endings produce generator potentials of sufficient amplitude to fire nerve impulses. But as the pressure of the ampulla is increased, the generator potential is driven progressively to the critical firing level, and impulses are discharged rhythmically and continuously. The main effect of a pressure increment is to increase the rate of rise and the amplitude of the generator potential, and, thereby, to increase the frequency of discharged impulses.

Progressive ablation of ampulla cell structures shows that the generator potential arises in, or close to, the pyramidal cells. The generator structures are excited effectively by either (1) an increase in internal ampulla pressure, that involves stretching; but not by (2) increasing equally and simultaneously the internal and external ampulla pressure, *i.e.*, by a pure pressure stimulus.

The Lorenzinian ampulla seems thus well suited to detect hydrodynamic pressure changes, caused by the fish's own movement or by external objects. In the dynamic phase, its sensitivity is about 11 impulses/second per meter.

Electron microscopy of electrical synapses in the crayfish. A. J. DE LORENZO.

Synaptic junctions in the abdominal ganglia of the crayfish were examined with the electron microscope. Particular attention was directed to axo-axonal junctions which exhibit physiological properties of electrical transmission. The lateral giant to motor junction is characterized by many post-junctional processes which invaginate into the lateral giant or prejunctional fiber. The appositional membranes are separated by a small space approximately 50–75 Å wide. This space is much smaller than that described in other synapses. Each of the appositional membranes is composed of a structural unit consisting of two electron-dense lines, about 25 Å thick, separated by a light zone about 30 Å wide. Occasionally, the unit membranes appear to fuse into a single electron-dense zone about 50 Å thick. Thus, in certain regions, the synaptic membranes exhibit structural modifications at variance with the unit membrane concept. Throughout the region of synapse, however, the synaptic cleft is uniformly 50–75 Å wide.

The axoplasm of the prefiber at the junction is structurally unremarkable, but occasionally rows of mitochrondria are evident. The post-fiber, however, contains large clusters of "synaptic vesicles" which are typically found in the presynaptic terminals of other synapses. Since these synapses have been shown to permit the flow of electrical current only in one direction, it is surprising to find an abundance of synaptic vesicles in the post-synaptic processes. This observation is particularly interesting in view of the current speculation regarding the role of synaptic vesicles in chemical transmission. The role of "synaptic vesicles" in a strictly electrical synapse remains unresolved.

Segmental or septal junctions were also examined. In this junction the synaptic cleft is quite large, often on the order of 400 Å. However, in regions where the pre- and post-fibers interdigitate, the fine processes are separated by clefts about 100 Å wide. It appears that the synaptic cleft in this junction is not uniform in dimensions. Thus, preliminary observations of two electrical junctions suggest differences in ultrastructural organization.

Reversible inhibition of metamorphosis in tadpoles of Amaroecium constellatum by calcium-free sea water. WILLIAM F. LYNCH.

Tadpoles of *Amaroecium constellatum* were taken through four washes of Moore's calcium-free sea water at a pH of 6.2 and were deposited in Stender dishes of the same medium where they became quiescent in a few minutes. All remained in the larval state during seven-hour periods of observation. (The normal maximum natatory period is one hundred minutes.) At the end of seven hours the tadpoles were washed in natural sea water and placed in Stender dishes of this medium. Twenty-four hours later they had formed ascidiozoids with well-developed pharyngeal regions and siphons. The internal organs had assumed the adult axial relationship. Tadpoles that were not removed from the first bath of calcium-free sea water had begun metamorphosis by seven hours, but those in the other baths had not. In the first washing dish metamorphosis had proceeded to the stage in which rotation of the larval axis had occurred, but the process ceased while the larvae remained in a contracted state. At a pH of 8.1 a similar inhibition of metamorphosis was noted, but this inhibition was no longer reversible when the exposure to calcium-free water lasted for nine hours.

Silicoflagellate populations in the plankton of Cape Cod area, past and present. RAMON MARGALEF.

In the superior layers of the sediment of Cape Cod and Buzzards Bays, skeletons of silicoflagellates are common. The following species of Siphonotestales have been recognized: *Dictyocha fibula* Ehrenb., including var. *pentagona* Schulz, *Distephanus crux* (Ehrenb.) Haeck., *Distephanus speculum* (Ehrenb.) Haeck., including vars. *pentagonus* Lemm., *regularis* Lemm. and *septenarius* (Ehrenb.) Joerg., and *Cannopilus binoculus* (Ehrenb.) Lemm. (probably a simple form of *Distephanus speculum*).

Skeletons increase in abundance from around 16 cm. below the top of all sediment cores towards the surface. Typical *D. fibula* and *D. speculum* make together 99% of the populations. As we approach present time, a shift, similar in both bays, is observed in the composition of populations. *Dictyocha fibula* is almost exclusively represented in the inferior levels. *Distephanus speculum* increases in a regular way from a negligible representation at 8–16 cm. below the surface of sediments up to make 50–60% of the skeletons of silicoflagellates present in the uppermost, soft, layer of the cores.

Dictyocha fibula is a species perhaps of more oceanic character and of rather warm environments; *Distephanus speculum* is more commonly reported from colder water with lower salinity. A differential dissolution of skeletons is unlikely, so that change in the representation of both species may reflect changes in the average composition of plankton accomplished over many years.

The size of the marine diatom Melosira sulcata (Ehrenb.) Kuetz. in the Cape Cod area. RAMON MARGALEF.

In natural populations of several species of *Melosira*, distribution of cells according to diameter shows definite peaks. Such species, all thick-walled, do not experience a noticeable reduction of diameter along successive cellular divisions. Their size distribution suggests the manifestation of a polymorphism.

Different morphs (peaks) seem to have a different selection value under definite environmental conditions and their proportions change in space and in time. Observations in Vigo (NW Spain) suggest that in *Melosira sulcata* low salinity and colder water are, in an independent way, favorable to the high diameter classes.

Forty-two hundred frustules of *Melosira sulcata* have been measured in 5 bottom cores taken in Buzzards Bay and 4000 in two cores obtained in Cape Cod Bay, at depths of 15–29 meters. Diameter ranges from 7 to 50 μ and distribution of cells by diameter offers an image similar to European populations, with peaks at 10, 12–13, 16–17, 20–21, 27, 36–37 μ. Differences exist between bays, between stations and between layers. Shifting of sediments by bottom dwellers does not disturb completely the historical record.

The superior sections of cores show notable parallelism. Considering three levels: a) about 8 cm. below the surface of sediments; b) top of consolidated sediment, ordinarily 3 to 4 cm. below surface; c) unconsolidated materials about 1 cm. below surface, we get the following average diameters: Cape Cod Bay: a) 18 μ, b) 16.7 μ, c) 17 μ; Buzzards Bay: a) 20 μ, b) 17 μ, c) 17.3 μ. The decrease of size, starting at an indeterminate time, is followed by a recent and slight increase. A tentative explanation may be acceptance of an increase of temperature followed by a recent cooling (or decrease of salinity). Salinity rather than temperature may explain the differences between bays.

Pigment composition and productivity as related to succession in experimental populations of phytoplankton. RAMON MARGALEF AND JOHN H. RYTHER.

A type of compound chemostat, made with serially-connected culture vessels, has been employed for the laboratory study of phytoplankton succession. After initial inoculation, fresh medium is flowed slowly through the system. Soon a steady-state is reached where cell density is independent of the initial state. Then flow can be discontinued and the following measurements made on each vessel: productivity (C^{14} assimilation), cell counts and pigment analyses. The rate of increase, r, in a given vessel is obtained by the expression $r = \ln\ [1 + (V/C)(C\text{-}B)]$, where C is the cell concentration in the vessel, B the concentration in the previous vessel and V the volumes of the vessel exchanged per unit of time.

In experiments with uni-algal (*Amphidinum*) and mixed (*Syracosphaera*, *Skeletonema* and *Chaetoceros*) cultures there was consistently observed a decrease in productivity and an increase in the ratio of optical density, 430/665, of acetone extracts of the pigments along the successive elements of the system representing stages of a succession.

Typical results with *Amphidinum* (22° C., 450 foot candles) in a series of three vessels are shown below. In this experiment pH reached 9.5 in some vessels and productivity was based on available bicarbonate only, there being a suspicion that tagged carbonate was not exchanged rapidly with bicarbonate or actually taken up by the algae.

(a) continuous illumination. D430/D665: 3.25, 3.80, 3.85; chlorophyll *a* (μg./10^6 cells): 3.67, 3.65, 2.25; r: 0.88, 0.39, 0.27; productivity (μg. C/10^6 cells/hr.): 1.06, 0.78, 0.37.
(b) 13 hours light/day. D430/D665: 3.55, 3.95, 4.74; chlorophyll *a*: 3.75, 2.70, 1.80; r: 0.78, 0.33, 0.16; productivity: 2.16, 1.86, 1.39.

Note the greater activity of cells subjected to the day-night rhythm.

Purification and properties of Limulus arginine phosphokinase. R. A. MORRISON, A. M. MORGAN AND G. W. DE VILLAFRANCA.

A method for the partial purification of arginine phosphokinase (APK) from the skeletal muscle of *Limulus polyphemus* was investigated. The bulk of the enzyme was precipitated from 50–100% ammonium sulfate saturation of the initial water extract. Dialysis of the residue against 0.001 *M* arginine, pH 8.7 and refractionation with ammonium sulfate between 65–90% saturation resulted in a fraction with a 6.6-fold purification and a 69% recovery. Further fractionation did not increase the specific activity but led only to a decrease in the recovery.

The reaction catalyzed by arginine phosphokinase was followed by assaying for free arginine and was run in the direction:

$$\text{ATP} + \text{arginine} \rightarrow \text{ADP} + \text{phosphoarginine}$$

Activity was found to be directly proportional to time, up to 10 minutes, and also to the protein concentration over a 5-fold range with the substrate concentrations used. The pH optimum is 8.6 and the temperature optimum is in the range of 15–18° C. When the reaction was allowed to proceed at 37° C., the activity was reduced to 50% of the maximum. Although a slight residual activity was found in the absence of added cations, activation occurred in the presence of the following cations in decreasing order: $\text{Mn} > \text{Mg} >> \text{Fe} > \text{Ca} > \text{Hg} > \text{Co}$; Cd inhibited. Optimal activity was noted when the ratio of arginine : ATP : Mg^{+2} was 1 : 1 : 1. When the Mg : ATP ratio was greater or less than one, APK activity decreased. The K_m value for arginine at pH 8.7, 25° C. is 2.52×10^{-3} *M*. Substrate specificity was tested with respect to creatine, glycocyamine, taurocyamine, lombricine, and arginine; phosphorylation occurred only with arginine. *Limulus* APK is very labile; it is easily denatured by alcohol, dilution, heat, and by standing at 5° C. Freezing, both in the presence and absence of cysteine, led to a complete loss of activity, while the same treatment in arginine buffer, pH 8.4, showed a 67.7% decrease in activity.

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Comparative aspects of active transport of D-glucose by in vitro preparations of fish intestine. X. J. MUSACCHIA AND S. S. FISHER.

In vitro preparations of everted intestinal sacs were used to measure absorption and transport of D-glucose in segments of intestine from a variety of fish; 33 scup, *Stenotomus versicolor;* 2 sea bass, *Centropristes striatus;* 3 tautog, *Tautoga onitis;* 6 toadfish, *Opsanus tau;* 8 eel, *Anguilla chrysypa;* 71 bullhead catfish, *Ameiurus nebulosus;* 2 perch, *Perca flavescens.*

Active transport of D-glucose was obtained in all the preparations from the fresh-water fishes, in four preparations from eels and in none of the preparations from any of the marine fish.

In a typical experiment, the everted segment of mid-intestine was filled and suspended in teleost Ringer's containing a specific concentration of sugar (5 and 10 mg.%). Preparations were incubated at fish body temperatures (22° to 24° C.) for 20, 30 or 40 minutes. The starting concentrations in the mucosal and serosal fluids were equal. During absorption and transport, sugar was moved against a concentration gradient and increased in concentration in the serosal fluid and concommitantly decreased on the mucosal side. Active transport was uniformly evident in the preparations made from catfish; starting with 10 mg.% glucose, approximately three-fold differences were obtained after 30 minutes. The mean values for 41 preparations changed to 23 mg.% glucose in the serosal fluid and 6.5% in the mucosal fluid. Comparable results were obtained where 5 mg.% glucose was the starting concentration and after 30 minutes, for an average of 30 preparations, glucose in the serosal fluid increased to 13 mg.% and decreased to 2.6 mg.% in the mucosal fluid.

A series of 33 preparations (5 and 10 mg.% glucose at 22° C.) made from scup showed mucosal absorption but no transport of D-glucose. Incubation at elevated temperatures, 37° C., showed very little enhancement of absorption.

Histological preparations showed that the intestinal mucosa retained its integrity during the experimental procedures.

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Some characteristics of active transport of sugars by intestinal segments of Ameiurus nebulosus. X. J. MUSACCHIA.

Absorption and active transport of D-glucose *in vitro* preparations of intestinal segments from the catfish, *Ameiurus nebulosus,* have been reported. In the present study the upper region of the midgut intestine was found to be more efficient in D-glucose transport. Average serosal/mucosal ratios (15 preparations) in 5 mg.% D-glucose were: upper segments, 8.37 ± 1.66; lower segments, 4.93 ± 1.69. At 10 mg.% D-glucose average S/M ratios were: upper segments (19 preparations), 4.74 ± 0.79; lower segments (21 preparations), 2.80 ± 0.40. Routine histological examinations showed shorter and more compact villi in the lower midgut regions, indicating a decrease in total mucosal surface area. Other differences, *viz.* cytological architecture of the mucosal epithelium, distribution of goblet cells, etc., were noted. Such features may have some bearing on the differences in absorption and transport of D-glucose.

A control series of 15 "blank" preparations, wherein the segment was incubated in teleost Ringer's minus the sugar, showed that the amount of glucose endogenously produced ranged from a non-detectable amount in the mucosal fluid to a mean fraction of 2 mg.% in the serosal fluid. These values had no effect in the ultimate determination of glucose.

Low temperatures, 0° to 2° C., inhibit absorption and transport of D-glucose. Preparations were incubated in 10 mg.% D-glucose at low temperatures for 20 minutes and S/M ratios were 1.18 ± 0.04. The same preparations were then incubated at fish body temperature, 22° to 24° C., and active transport was obtained, the S/M ratios being 2.64 ± 0.24. Four preparations were made anoxic with 100% nitrogen for 30 minutes and little or no inhibition of D-glucose transport resulted.

Other sugars, D-xylose, 10 mg.% and 20 mg.%, and D-fructose, 30 mg.% and 50 mg.%, were not transported.

Aided by grant funds from Saint Louis University, Cancer Research Institutional Committee and a Grant-in-aid from the Sigma Xi-RESA Research Fund.

Further studies on the protoplasmic contraction of the marine alga, Chaetomorpha Linum Kützing. W. J. V. OSTERHOUT.

The following experiments form a continuation of the reports published in this Bulletin in 1955 and 1959 and the present *Chaetomorpha* resemble those described in 1959. The purpose is to determine what substances cause the protoplasmic contraction of dead *Chaetomorpha* cells.

A string of living cells was placed in filtered sea water at 50° C. for five minutes. It was then transferred to filtered sea water at 25° C. for one-half hour, after which it was rinsed in distilled water, wiped, and placed in a test solution at 25° C. The cells were at once examined under the microscope and at the start no contraction was detected. But subsequently the protoplasm began to contract. The rate and the degree of contraction in various solutions were compared as follows.

The following solutions are arranged in the decreasing order of their effective action on protoplasmic contraction. HCl, H_3PO_4, both at pH $1.6 > KH_2PO_4$, NaH_2PO_4, both at pH $4.5 >$ phosphate buffer mixture at pH $6 >$ glass-distilled water at pH $6 >$ phosphate buffer mixture at pH $8 >$ borate buffer mixture at pH $8 > Na_2HPO_4$, K_2HPO_4, both at pH $9.5 >$ borax at pH 9.5. Control in filtered sea water showed no contraction, even after 40 hours.

Very rapid and severe contraction occurred in acid solutions at pH 1.6, but much slower and less severe contraction occurred in alkaline solutions at pH 9.5, showing a great difference between the acid and the alkaline solutions. There was less difference between the phosphate and borate buffer solutions at the same pH value. Five-hundredths M and 0.01 M buffer solutions behaved alike. Sodium and potassium behaved alike.

The contraction was reversed in NaOH solutions at pH 13.

These results indicate that the protoplasmic contraction was brought about primarily by H^+ and secondarily by PO_4^{---} and that the contraction was reversed by OH^-. They may suggest that in a living cell the acids and phosphates formed by metabolism may cause protoplasm to contract, which is reversed by the production of an alkaline substance. This may partly account for the protoplasmic movement.

Experiments with triphosphates will be reported later.

The effects of chloramphenicol on cleavage of Arbacia eggs. Edward E. Palincsar.

The effects of chloramphenicol on early cleavage of Arbacia were examined to determine the effect of possible inhibition of protein synthesis on the structures and dynamics of cell division. *Arbacia* eggs and sperm were treated with various concentrations (0.2–2.0 mg./ml.) of chloramphenicol in sea water. Cleavage was delayed up to four hours depending upon the concentration, temperature and time of immersion into the chemical. The concentration for blockage of division was 1.5–2.0 mg./ml. Temperatures were experimentally varied from 20–30° C.

Thirty-minute pretreatments of unfertilized eggs and sperm in 0.5–1.0 mg./ml. yielded up to a 34-minute delay in the first cleavage. Fertilization was not affected, but if both treated eggs and sperm were used in fertilization, there was a marked inhibition of cleavage rate and failure to survive past the morula. Five- and 10-minute treatments of the zygotes in 1.0 mg./ml. of chloramphenicol suggested that the period most sensitive to subcritical concentrations is prior to prophase. Blockage in 1–2 mg./ml. is reversible and the inhibition appears to be a freezing of cleavage at the stage when treated. Once the block is reversed, the eggs proceed with the stage at the time of arrest. Using a Warburg respirometer, preliminary data were obtained which indicate no effect of inhibitory concentrations (0.2–0.5 mg./ml.) on oxygen consumption. Subcritical concentrations (0.2–0.5 mg./ml.) did not appear to affect the microscopic appearance, solubility, response to enzymes (trypsin, pepsin, chymotrypsin, ribonuclease) or RNA distribution (azure-B, methyl green-pyronin) of the mitotic apparatus, which was isolated using the dithiodiglycol method. Cytokinesis is unaffected in subcritical concentrations, but there is a marked inhibition of membrane elevation. These preliminary results suggest that the proteins of the mitotic apparatus might be synthesized prior to prophase, since that is where this depressant of protein synthesis has its greatest effect. This possibility is being investigated with isotopes.

The effects of 8-azaguanine and chloramphenicol on the regression-replacement cycle of hydranths. Edward E. Palincsar and Joan S. Palincsar.

Substances believed to inhibit protein synthesis and nucleic acid metabolism were used to treat *Obelia* colonies, to determine the phase of the hydranth cycle which might be most affected and thereby to gain some insight into the cycle transformation. *Obelia* colonies were cultured in aerated sea water (Crowell's method) containing concentrations of 8-azaguanine ranging from 10^{-4} M to 10^{-3} M, or chloramphenicol concentrations ranging from 0.1 mg./ml. to 1.0 mg./ml. Temperature was constant and observations and counts were made up to 14 days. Concentrations of chloramphenicol above 0.3 mg./ml. and concentrations of 8-azaguanine above 4×10^{-4} M were toxic, bringing about regression of the hydranth to the bud stage and eventual disintegration. Eliminating the chemicals resulted in recovery by new upgrowths from the stolon, which thus appeared to be the most resistant. At the lowest concentrations of both chemicals, peculiar, large, elongated, free stolon-like structures grew from the ends of the uprights and occasionally from the sides. These growths were twice the width of a bud, and from 4 to 10 times the length by the second day. The perisarc was not annulated and there was no visible differentiation except a narrowing of the stalk. After four days, small but otherwise normal hydranths differentiated on the ends and sides of the growth. These preliminary results suggest that stages involving differentiation and organization are most sensitive to the chemicals, and that proliferation phases are least inhibited. The possibility of uncoupling growth and differentiation is being further investigated. The chemicals also delayed the regeneration time of cut stems of *Tubularia*. Histochemical studies showed no changes in distribution of RNA and DNA, increased concentrations of succinic dehydrogenase in active hydranths and areas of growth, and increase in calcium concentration during late regression.

The role of moisture and illumination on the expression of the rhythmic behavior of the diatom, Hantzschia amphioxys. JOHN D. PALMER.

The diatom, *H. amphioxys,* surfaces on the sand flats in Barnstable Harbor, Mass., at the time of low tide and migrates downward into the sand when the area is reflooded. In 1951 Fauré-Fremiet reported this rhythmic behavior to persist for six days away from tides in laboratory cultures.

Experiments were conducted during the summer of 1960 to see if any environmental influences on this rhythm could be detected. At high tide sheet metal dams were positioned over previously delimited patches of cells, to retain water in these areas as the tide receded. The resulting cover of water prevented the appearance of algae on the surface, the algae appearing, however, within 30 minutes following draining of the dammed area. The natural flooding tide, or artificial flooding, caused the organisms to migrate downward. In the laboratory, the rhythmic migrations failed to occur under conditions of either constant flooding or drying beyond some critical degree.

Darkness also inhibited the rhythm. Opaque shields placed on the sand before the diatoms normally come up prevent the vertical migration during low tide, and similarly, shields placed at low tide induce the return of the organisms into the sand. That light is the factor involved here is evident from the observation that movements proceed normally under a transparent covering.

Diatoms observed in "preference-chambers" in the field, but isolated from the tidal change, move into the illuminated portion at low tide and into the darkened portion in synchrony with the downward migration of the natural population.

These studies indicate that there is a tidal rhythm in the sign of the phototactic response, it being positive during the time of low tide, and that the expression of the rhythm is influenced by tidal water movements.

Electron microscopic studies of Fucus vesiculosus cytoplasm in summer and winter. JOHNSON PARKER AND DELBERT E. PHILPOTT.

Longitudinal hand sections of *Fucus* midrib 2 inches from the tip were made in January, fixed in buffered OsO_4 at 0° C., dehydrated, embedded in 1:3 methyl and butyl methacrylate, and sectioned longitudinally with a diamond knife. Chloroplasts were found clumped together in winter, but lamellae were clearly visible with six layers each (not four as reported in the literature). In cells with slightly shrunken contents, cytoplasm tended to adhere in small strands to the wall, and as a result of this the wall was sometimes slightly expanded, revealing about 25 layers. Good OsO_4 fixation in June was difficult, possibly because of the state of hydration of the cytoplasm or because of its permeability, a situation which might be related to the lower resistance of *Fucus* to sub-zero temperatures in summer than in winter. $KMnO_4$ at 5% for 10 minutes at 22° C. by Mollenhauer's method gave better results for summer material; chloroplasts were observed resembling those of winter except that they were not clumped. Vacuoles in cytoplasm were more evident in summer and the cytoplasm as a whole was more electron-dense in winter. Cytoplasm clearly passed from cell to cell in medullary elongated cells, as has long been suspected. These connections are relatively massive intrusions of cytoplasm in comparison to the fine strands penetrating the pores of *Macrocystis pyrifera* (brought from the West Coast) sieve plates. Strands in *Macrocystis* appeared to be extensions of endoplasmic reticulum.

Histological investigation of the central nervous system of Clymenella torquata. MABEL C. PATERSON.

In order to understand the significance of regeneration studies in *Clymenella torquata* a more complete knowledge of the histology was considered necessary; consequently an investigation of the central nervous system was undertaken. Anterior regions were serially cut, frontally and sagittally, and stained with a modification of the Holme's buffered silver nitrate technique. The central nervous system is similar to previously described polychaete systems, and consists of a ventral nerve cord which terminates in an enlargement, the subpharyngeal ganglion. This ganglion is united with the paired suprapharyngeal ganglia, located on the dorsal aspect of the pharnyx, by a pair of circumpharyngeal commissures which encircle the pharynx. A number of branches arise from the circumpharyngeal commissures. Each suprapharyngeal ganglion consists of two lobes and receives two major fiber tracts. One of these enters antero-medially and connects with a group of special sensory cells located on the dorsal head plaque, the other enters ventro-laterally and innervates the stomadeal region. The two suprapharyngeal ganglia are connected midway by a bridge of fibers.

The nerve cells within each suprapharyngeal ganglion are peripherally located and appear to be collected in two main groups, anteroventral and dorsal. The fibers of the circumpharyngeal commissure enter the lateral side of each ganglion and therefore separate the two groups of cells. The majority of the cells are of medium size and appear to be bipolar. Their nuclei are round and centrally located. They usually contain one, or occasionally two, eccentrically placed nucleoli. The cytoplasm is granular and some indication of neurofibrils has been observed.

Currently the cellular nature of the subpharyngeal ganglion and the ventral nerve cord is being investigated, as well as possible neurosecretory activity.

Histological investigation of the nephridia of Clymenella torquata. MABEL C. PATERSON AND CARRIE R. KREWSON.

The anatomy and histology of the nephridia of the polychaete *Clymenella torquata* were studied. Four pairs of large nephridia are located laterally in the coelom in segments seven through eleven. Each nephridium occupies portions of two segments, the nephrostome lying in front of the segmental septum and the remainder of the organ immediately posterior. Nephridial openings are observable ventrally at the base of each parapodium.

A plastic reconstruction of a nephridium showed that the organ consists of a funnel-shaped nephrostome opening to the coelom, a long, slightly-coiled, unbranched tubule extending from the nephrostome, a bladder, and a short, straight neck region leading to a nephropore.

The nephrostome is composed of a single layer of ciliated columnar epithelium with a distinct basement membrane. The everted lips of the nephrostome appear in close association with a branch of the sub-intestinal blood vessel. The tubular portion consists of a single layer of irregularly-shaped, ciliated columnar epithelium with a basement membrane; the cytoplasm is densely granulated and highly vacuolated. The bladder and neck regions are formed of a single layer of ciliated squamous epithelium. At the tubule-bladder junction a large tuft of cilia was noted. Ova and sperm packets were observed in the tubule and bladder, suggesting the organ functions in gamete release.

Preliminary histochemical studies indicated alkaline phosphatase localizations in large, sharply-defined granules packed uniformly throughout the cytoplasm in the tubule. No positive alkaline phosphatase reaction was observed in the nephrostome; the bladder showed a positive reaction only at its junction with the tubule. The ciliated inner border of the entire organ is PAS-positive. PAS-positive material was also observed in the basement membrane and in small granules scattered throughout the cytoplasm, particularly in the tubular area. These PAS-positive granules are not comparable to the alkaline phosphatase-positive granules observed in the tubule.

Further electron microscopic observations on the sperm of Limulus polyphemus. Delbert E. Philpott.

Previous description of the ultrastructure of the sperm of *Limulus polyphemus* reported the head of the sperm as having an acrosome cap and a small "axial" body at the anterior end. An axial core was also seen, traversing the head centrally from the small axial body to the region of the centriole in the distal end of the head. At the time of the first investigation it was not possible to determine if the posterior spiral, consisting of six closely adhering turns, continued up the axial core to the axial body or if the axial core was a process unto itself. Embedding the sperm in epoxy resin and making numerous sections revealed that the posterior spiral does continue up the axial core to the axial body and is thus a continuous structure.

The use of carbon for single molecule visualization. Delbert E. Philpott.

The method of Cecil Hall for the visualization of individual molecules has been modified in the following way. After the specimen has been sprayed on mica and shadowed with platinum according to his method, carbon is evaporated down onto the specimen from 90° in the place of silicon monoxide. This film is now sufficiently strong to eliminate the next step of flooding the surface with collodion. This saves the time involved in the last step, but more importantly the replacement of silicon monoxide plus collodion as a supporting film increases the brilliance and contrast of the specimen image in the electron microscope. In fact, in some instances the latex particles have been left out of the specimen to be sprayed, both because it was felt advantageous to keep the specimen pure, and because all of the material which is visible on the final print is also visible on the fluorescent screen during specimen scanning. After the carbon evaporation the mica must be placed at once into distilled water in order to float off the prepared specimen. This results in a silver film which easily floats off without the aid of score marks or any other help. However, waiting 24 hours nearly always results in unstrippable films. This stripped film is picked up on 200-mesh copper sheet cut to the size of the floating specimen film, and brought up from underneath, thus mounting the specimen. After blotting the grid material on filter paper, grids are punched out in the usual way for examination. The final result will not be a great deal different from the original and clever method referred to above, but the increased ease and simplicity with which the method can be applied should prove an asset to others, as it has in this laboratory.

Electron microscopic observations of the starfish eyespot. D. E. Philpott and A. B. Chaet.

A preliminary investigation of the red eyespot located on the short sensory tentacle at the tip of the arm of the starfish, *Asterias forbesi*, was undertaken by means of electron microscopy. The eyespots were dissected from the animal, fixed in osmic acid sea water, embedded in methacrylate and sectioned.

Each of the many eye cups found in the eyespot consists of a cone-shaped layer of red granules, the opening of which is directed towards the outer surface. Electron micrographs indicate two different-sized granules. The smaller, more osmiophilic pigment granules measure about 0.2×0.1 micron in diameter, whereas the larger ones are 1.3×0.8 microns in size. The pigments of the eyespot, B carotene and esterfied astaxanthine, may be represented by the two sizes of granules.

Within the cup two different structures are visible. The one filling the posterior four-fifths of the pigment cup appears quite uniform in nature and is connected at intervals to the lateral walls. The second structure, which is found in the anterior portion, as a layer lying at right angles to the cup's axis, appears to be identical to the outer ectodermal surface of the eyespot itself. This layer as well as the outer surface is about one micron in thickness, and both are covered with micro villi. The layer within the pigment cup may have arisen as part of an invagination of the outer surface, thus explaining the presence of the micro villi which are pointed into the cup. Future attempts will be made to determine if this ectodermal-appearing layer represents a potential lens, and whether the posterior four-fifths of the cup represents neural conducting material.

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Comparative ecology of two species of intertidal amphipods: Talorchestia megalopthama and Orchestia agilis. SARA J. PLATZMAN.

In an intertidal environment humidity is obviously a central factor in determining the distribution of organisms. The intertidal animals would therefore be expected to show adaptations for reduced humidity. Experiments were performed on *Talorchestia megalopthama* and *Orchestia agilis*, two species of intertidal amphipods found near Woods Hole, Massachusetts, at 26.5° C., the approximate summer temperature of this area. At 0% R.H. *Talorchestia* survived for an average of 44 and *Orchestia* 33 minutes. By comparison the members of the wholly marine genus *Amphithoe* survived an average of 13.5 minutes and *Armidillidium vulgare*, a terrestrial isopod, was found by Waloff to live for 429 minutes in 0% R.H. Therefore the R.H. tolerances of *Orchestia* and *Talorchestia* represent a modification toward a land habitat.

The slight difference in humidity tolerance between *Talorchestia* and *Orchestia* is not large enough alone to restrict them to different intertidal habitats. *Orchestia* is found under moist sea-weed at the high water level and is seldom, if ever, present on the surface of the sand. In contrast, the adults of *Talorchestia* live, during the day, beneath 1–5 inches of sand: the lower limit of distribution is 3–4 feet above the water mark of all phases of the tide. Unlike *Orchestia*, they are not confined to the high-water mark. At least part of the populations of *Orchestia* are active day and night. *Talorchestia*, however, is only active on the surface, near the water, at night, although a few juveniles can be found hopping on the surface near the water or in the sea-weed during the day. These observations indicate that behavioral and distributional differences between these two closely related species seem more important than humidity in differentiating their niches.

This work was done as part of the student training program of the Marine Ecology Course.

Analysis of photodynamic effects in lobster neuromuscular preparations. J. P. REUBEN.

Muscle fibers soaked for brief periods in 10^{-4} M acridine red, neutral red, or sevron blue responded to illumination with white light by 2 to 10 mV changes in membrane potential. The potentials were of either sign and, when hyperpolarizing, they were reversed by increasing the negative resting potential somewhat more than 15 mV with intracellularly applied hyperpolarizing currents. The membrane resistance, which was increased in the dark after application of the dyes, was lowered during the period of illumination and for some seconds thereafter. The directly evoked responses of the muscle fibers, which were converted from graded responses to spikes by the dye, were reduced during the illumination. Both the excitatory and inhibitory postsynaptic potentials (p.s.p.'s) were also reduced during the irradiation. Return of the amplitudes of the responses to their initial values occurred in the tempo of the return of the membrane resistance.

The frequency and amplitudes of both excitatory and inhibitory miniature p.s.p.'s were, however, greatly enhanced during illumination of the dye-treated preparations. Picrotoxin, which blocked the inhibitory miniature p.s.p.'s, did not block hyperpolarization of the fibers by irradiation. The increased miniature activity appears to be caused by photodynamic effects on the presynaptic nerve fibers. In the muscle fibers, however, the dyes appear to sensitize a photodynamic conductance change in a component of the membrane that is independent from the electrically excitable or the synaptically activated membrane components. This effect may be analogous to the conversion of previously inert membrane to a chemically excited one in denervated vertebrate muscle fibers. The increased conductance of the photodynamically activated membrane shunts and decreases both the directly evoked responses and the p.s.p.'s.

Electrotonic connections between lobster muscle fibers. J. P. REUBEN.

Depolarizing or hyperpolarizing currents that are applied intracellularly to one (the "proximal") muscle fiber produce changes of the same sign, but of lower amplitude and changed form, in the membrane potential of other ("distal") fibers. The electrophysiological data on the size and form of these electrotonic potentials indicate the occurrence of anatomical structures which form high resistance connections between the two approximately symmetrical resistance-capacity networks of the two muscle fibers. The electrotonic potentials in the distal fiber were attenuated as much as 10-fold in many fibers, but in some there was only 2- to 3-fold attenuation. The greater the attenuation, the more marked was a phase shift between the applied current and the electrotonic potential in the distal fiber. With the larger phase shifts, the potential in the distal fiber continued to grow after the cessation of a brief pulse of current. All-or-none spikes, which were directly evoked in one fiber of procaine-treated preparations, also caused potentials in other fibers, the degree of their attenuation being related to the degree of attenuation of applied pulses. The connections did not exhibit rectification, flow of current apparently being symmetrical in the two directions.

These data and experiments on a model equivalent circuit indicate that the muscle fibers are connected by a link which has a resistance some 3 to 10 times higher than that of the individual fibers. Whether the interconnections are few or numerous between a pair of fibers could not be determined because lobster muscle fibers have a large length constant. There appear to be mutual interconnections in groups of adjacent fibers. However, the electrophysiological evidence of electrotonic connections cannot resolve whether they are formed by syncytial or ephaptic links or by both. In some cases, no electrotonic effects were observed between immediately adjacent fibers, indicating the absence of connections between these pairs. In *Carcinus* and *Cancer* muscles the findings are different.

Actions of cesium ions on the electrically excitable membrane of lobster muscle fibers. J. P. REUBEN AND H. GRUNDFEST.

As is also the case for neuromuscular transmission, short-term exposure of the fibers to Cs^+, with or without removal of K^+, did not affect the electrically excitable membrane. Soaking the preparation for 10 hours or more in K^+-free Ringer's solutions containing 15 to 50 meq./l. of Cs^+ did not markedly change the membrane potential or resistance from their values in K^+-free solutions without Cs^+. Direct stimulation still produced small graded responses, although the large excitatory postsynaptic potential of the Cs^+-soaked preparations caused a large spike-like response. Profound changes in the ionic conductance mechanisms of the electrically excitable membrane could nevertheless be shown on additional treatment of the preparations with various agents. Thus, while increasing external K^+ caused depolarization, the change was only about 30 mV/decade change in K^+. On changing the medium from zero K^+ to 30 meq./l., the resistance fell only 3- to 4-fold, while in untreated muscle fibers the change is about 100-fold. Unlike untreated preparations, those soaked in Cs^+ tolerated exposure to a Ca^{++}-free medium for long periods. On diminishing the external Ca^{++}, the directly evoked responses increased in size. However, there was also a large, reversible decrease in membrane resistance, though only a small decrease in resting potential.

The addition of procaine (10^{-3} to 10^{-2} w/v) to normal preparations converts their graded responses to brief, all-or-none spikes. In Cs^+-soaked preparations, application of the drug caused indefinitely prolonged spikes, that were followed in some experiments for more than 30 minutes and terminated only by applying strong hyperpolarizing currents. The conductance during the spike was up to 20 times higher than that of the resting membrane.

The overt effects of Cs^+ in general resemble and are synergistic with those of Ca^{++}, but they are more extreme under some experimental conditions and less so under others. These similarities and differences indicate that Cs^+, like the alkali earth ions, has several modes of action. They also indicate that the several molecular mechanisms which are presumed to underly these effects are independent to some degree.

Further analysis of the conversion of graded to all-or-none responsiveness in the electrically excitable membrane of lobster muscle fibers. J. P. REUBEN AND H. GRUNDFEST.

The normal graded activity of lobster muscle fibers can be converted to all-or-none by various agents. These effects can be analyzed in terms of the various changes of ionic conductances and electrode properties of the electrically excitable membrane (see Grundfest, this issue).

The conversion by cocaine (10^{-6} to 10^{-5} w/v) and procaine (10^{-3} to 10^{-2} w/v) occurs with only small changes in resting membrane potential and resistance. The spikes are brief compared with those produced by alkali earth or onium ions. The drugs therefore probably only decrease the outward current during the response, by inactivation of K^+-conductance. Cocaine in higher concentrations abolishes activity, probably by also blocking inward current. In contrast, the conversion of the membrane to all-or-none responsiveness by d-tubocurarine is associated with hyperpolarization and increased resting membrane resistance. Thus, this drug also exerts another of the actions of the divalent alkali earth ions, block of resting K^+-conductance. However, enhanced Na^+-conductance does not develop, and the spikes are brief. Methylene blue, sevron blue, acridine red and neutral red appear to act in the same way as does d-tubocurarine. Procaine, in combination with other agents (Ba^{++}, Cs^+, phenethylamine derivatives, or serotonin-like compounds), causes spikes that may last for 30 minutes or more. Thus, the combined action of the agents also blocks Na^+-inactivation to an extreme degree.

Procaine and d-tubocurarine convert all-or-none responses of other electrically excitable membranes to graded activity. Thus, in their actions on different cells, various agents may have "excitant" or "depressant" effects, depending upon the conditions that prevail in the specific membrane. The nature of the effects probably derives from the degree and the critical ratios to which the different factors of electrical excitability participate in each response, and from the degree in which each component is affected by the various agents. The latter variable is probably determined by different details of molecular structure that are as yet unknown.

Inhibitory and excitatory miniature postsynaptic potentials in lobster muscle fibers. J. P. REUBEN AND H. GRUNDFEST.

Miniature potentials of synaptic origin occur spontaneously in lobster muscle fibers, due to activation of the inhibitory as well as the excitatory synaptic membranes. The inhibitory miniatures may be of either sign, depending on electrochemical conditions. Both activities are probably due to summation of transmitter actions of many presynaptic terminals. Inhibitory miniatures of up to 1 mV have been observed and the excitatory tend to be still larger. The inhibitory activity is abolished by picrotoxin and the amplitudes of the excitatory miniature potentials are then greatly augmented. The frequency and amplitudes of the miniature potentials are modified by applying various agents to the preparation. NH_4Cl (15 meq./l. or more) at first causes an increase in the frequency of both types of miniature potentials. Later, both are few in number, but large and prolonged. When the inhibitory are eliminated by picrotoxin, the excitatory potentials grow to very large amplitudes. Large, spontaneous potentials, principally or exclusively of the excitatory variety, are induced after serotonin. The potentials occur independently in different fibers of the same muscle, though all are innervated by the same axon. Thus, the independent origins of the spontaneous potentials indicate that they probably occurred independently of an axonal spike, and that the transmitter actions occurred independently in different presynaptic terminals.

Presence or absence of miniature potentials provides a criterion for the mode of action of various agents on the different components of the neuromuscular system. Thus, a very powerful blockader of lobster neuromuscular transmission, the purified dinoflagellate "clam poison," does not eliminate either variety of miniature potentials, nor the larger potentials induced by NH_4Cl. Therefore, the poison must block arrival of the spike at the presynaptic terminals, while activity of the latter is unimpaired.

The action of cesium ions on neuromuscular transmission in lobster. J. P. REUBEN AND H. GRUNDFEST.

Addition of Cs^+ (15 to 50 meq./l.), with or without removal of K^+ in *Homarus* Ringer's solution, had no apparent effect on short-term exposure of the preparation to this ion. Profound alterations were produced, however, on soaking the preparations for 10 to 20 hours in K^+-free solutions containing Cs^+. The excitatory and inhibitory postsynaptic potentials (p.s.p.'s) of the muscle fibers were greatly augmented when recorded in a K^+-free medium, with or without Cs^+. A single stimulus to the excitatory axon evoked a large, spike-like potential and a vigorous twitch. The i.p.s.p. evoked by a single stimulus to the inhibitory axon was maximal, or nearly so. The degree of facilitation of the i.p.s.p.'s was correspondingly small.

The sensitivity of Cs^+-treated fibers to GABA was about like that of untreated preparations. Thus, the inhibitory synaptic membrane had not become more sensitive to this drug. The reversal potential was unchanged and the augmented i.p.s.p.'s could not have resulted from changed electrode properties of the synaptic membrane. The reversal potential of the e.p.s.p.'s was between 20 and 30 mV, inside negative, and probably had not been changed by the ion, but a direct test for the chemical sensitivity of the excitatory synaptic membrane was not available. It is unlikely, however, that the augmentation of both p.s.p.'s by Cs^+ was due to actions on the presynaptic terminals, increasing the transmitter action of both.

When K^+ was present in the medium, the p.s.p.'s were markedly reduced in size. The decrease of the i.p.s.p. was directly related to the decreased membrane resistance and the changed membrane potential of the muscle fibers. The reduction in the e.p.s.p.'s was much greater. When the external K^+ was double that of *Homarus* Ringer's solution the membrane resistance had decreased 3- to 4-fold, but the e.p.s.p.'s were diminished about 10-fold. The effect of K^+ on the e.p.s.p. therefore indicates that K^+ blocks competitively the Na^+ channels opened during activity of the synaptic membrane.

Properties of indefinitely prolonged spikes of lobster muscle fibers. J. P. REUBEN, R. WERMAN AND H. GRUNDFEST.

When treated with various agents (Reuben and Grundfest, this issue), lobster muscle fibers produce directly evoked responses that are spikes with amplitudes up to 120 mV and durations of more than 30 minutes. The membrane conductance during the response was increased 3- to 20-fold. Responses with conductances in the lower part of this range could be terminated by applying strong inward currents. The termination was associated with an increase in membrane resistance, and the change in potential had a time course resembling that of hyperpolarizing responses. In spikes with high membrane conductance, the potential returned to its plateau value even after strong inward currents had hyperpolarized the membrane for several seconds. This return may have been a manifestation of anodal break excitation.

Spontaneous termination of the spike was frequently associated with up to 15 mV hyperpolarization, lasting 15 seconds or more. In other cases it occurred through stages of intermediate plateaus, each lasting several seconds. Low frequency oscillations between metastable states often occurred and sometimes lasted more than 10 minutes. The durations of the individual oscillations varied. At different times during a single sequence their forms resembled those of vertebrate cardiac potentials, "upside-down" potentials, or hyperpolarizing responses. They ranged in amplitude from less than 1 mV to about 50 mV.

These different effects are ascribable to the operation of different combinations of the various ionic conductances and the consequent electrode properties of the electrically excitable membrane (Grundfest, this issue). Local changes in these relations, which probably reflect non-uniformities in different regions of the membrane, add further complications. Their various manifestations in lobster muscle fibers suggest a possible mechanism for "pacemaker" activity in other types of cells.

Further studies on the biochemical effects of x-radiation on Tetrahymena pyriformis. JAY S. ROTH, ELEANOR HENRY AND CAROL WIERCINSKI.

Three-day cultures of *Tetrahymena pyriformis* W, grown in proteose-peptone plus yeast extract, were collected by gentle centrifugation and thoroughly washed. Cells were suspended in water or buffer and irradiated with from 300,000–600,000 r. In some cases cells were irradiated in the presence of metabolites, or metabolites were added immediately after irradiation.

Growth studies using an inoculum of approximately 2×10^5 cells indicated that no cells survived 600,000 r. With lower doses there was a lag period in the growth of irradiated cells, the length of the lag being roughly proportional to the dose, but thereafter irradiated cells grew at the same rate as controls. When cells were irradiated with up to 600,000 r in 0.05 *M* phosphate buffer, pH 7.4, there was either a slight stimulation of respiration or no change. However, irradiation in water resulted in the depression of respiration from 50–90% at 600,000 r. Addition of thioctic acid (0.5 mg./ml.) to cells during or immediately after irradiation with 300,000 r had no significant effect on respiration, nor did it appreciably affect the increase in respiration observed when irradiated cells were treated with 0.05 *M* pyruvate. Lactate (0.05–0.2 *M*) stimulated the respiration of control cells, and the oxidation of lactate by irradiated cells was increased over that of controls by from 20–100% as long as two hours post-irradiation with 300,000 r. The amount of increase was dependent on the time post-irradiation and the concentration of lactate. The oxidation of lactate was depressed, nevertheless, in a cell-free homogenate of *Tetrahymena* irradiated with 300,000 r, as well as in a homogenate prepared from irradiated cells.

The effects observed appear to be explained best on the basis of an increase in permeability of the cell membrane, and possibly mitochondrial membranes to lactate and pyruvate after irradiation. (Supported by a grant from the U. S. Atomic Energy Commission.)

X-ray-induced mitotic delay in the Arbacia egg. RONALD C. RUSTAD.

Since multipolar divisions appear to define damage to a system controlling the multiplication of asters, further studies on the parallelism between the induction of multipolar spindles and mitotic delay have been conducted. A pre-fertilization recovery period reduces both effects in irradiated eggs. The same mitotic stage (early streak) is maximally sensitive to the induction of both effects. Direct tri- or quadripolar second divisions indicate that the effect is general and not a special property of fusion nuclei.

The preparation for a multipolar division includes a previously undescribed mitotic stage: a double streak.

The experimental induction of polyspermy has been used to analyze the additivity of x-ray-induced mitotic delay. The presence of several irradiated sperm neither lengthens nor shortens the period of mitotic delay. When unfertilized eggs are irradiated and then made polyspermic, some of the eggs divide sooner than monospermic ones.

The division cycle of *Arbacia* eggs becomes insensitive to the induction of mitotic delay by either acridine orange or irradiation at approximately the same time. Treatment of sperm with either acridine or x-rays extends the x-ray-sensitive portion of the mitotic cycle, which corresponds cytologically to early streak, the period of multiplication and separation of the centrioles.

Low dose x-irradiation and the possibility of accelerated root growth. B. P. SONNENBLICK AND JOHN KEOSIAN.

It would be of theoretical and practical significance to determine definitively the existence of, and then induce as desired, the so-called "stimulating" effect of radiation on intact living organisms. An influence has been claimed by some earlier investigators and sporadically by recent ones, but others deny its existence. Would exposure of bulbs to doses circa 100 r influence subsequent rooth growth? *Allium* bulbs of heterogeneous origin (heterogeneity was desired) were exposed in exploratory tests to x-rays of 85 and 182 kvp, dose rates of 45 r and 350 r/minute. Germination began 12 hours later and test temperatures recorded from

24.1–26.3° C. Periodic observations were made and after 14 days all roots removed and their lengths measured. Almost 11,000 roots from approximately 400 irradiated and unexposed bulbs have been measured.

The results are equivocal. It is possible to record with doses of 50–100 r an accelerative influence, or the absence of such, according to the criterion used. Criteria may be (a) total number of roots, (b) average root length, (c) percentage of roots surpassing an arbitrarily chosen length, (d) percentage of bulbs germinating. In a given experiment an effect may be indicated by one or more of the criteria but not by others. Employing criteria (b) and (c) above, it would appear that low dose irradiation could have stimulated root growth relative to controls in two of six tests. It will be interesting to note future results if some variables in our complex system, or with other projected biological material, are eliminated.

Duration of the experiment is an important consideration. Roots from exposed bulbs may, during the first week, manifest an accelerated growth rate which might be regarded as stimulation were the test ended at that time. Control roots can often bypass the others in length during the second week. Early acceleration may thus be a spurious phenomenon.

Comparative effects of x-ray and ultraviolet radiation of gametes on the developing sea urchin Arbacia. CARL CASKEY SPEIDEL AND RALPH HOLT CHENEY.

Effects of x-ray and 2537 Å ultraviolet (UV) radiation of Arbacia gametes were analyzed by observing the subsequent stages of development after mixing various combinations of radiated gametes. In addition to fertilization of normal eggs with normal sperm, 8 gamete combinations were studied: (1) x-rayed eggs with normal sperm, (2) UV-rayed eggs with normal sperm, (3) normal eggs with x-rayed sperm, (4) normal eggs with UV-rayed sperm, (5) x-rayed eggs with x-rayed sperm, (6) UV-rayed eggs with UV-rayed sperm, (7) x-rayed eggs with UV-rayed sperm, (8) UV-rayed eggs with x-rayed sperm. Radiation doses ranged from mild to severe. Fertilization occurred with all gamete combinations. Development was watched from the moment of insemination to the larval pluteus stage. Time-lapse cinephotomicrography recorded early viscosity changes and the sequence of protoplasmic movements culminating in irregular cleavages.

Certain differences in the progeny resulting from the 8 combinations of radiated gametes were ascribed to differences in penetration of the two kinds of radiation, deep with x-rays and shallow with UV-rays. Graded quantitative results, based upon both amount of developmental retardation and degree of injury, were more uniform after graded x-ray dosages than after graded UV exposures. Greater variation in UV-rayed egg experiments resulted from the different amounts of radiation that reached the near (to UV source) and far sides of the exposed eggs, combined with the variable position of the nucleus in individual eggs. Differences in radiation penetration also affected comparative radiosensitivity. Thus a short exposure of sperm (small-sized cells) to UV-rays caused developmental retardation and injury equal to a longer exposure of eggs (large-sized cells), 4–16 times as long. In contrast an x-ray dose to sperm was equal to a dose of only 1.5–2 times as much to eggs.

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Developmental potential of the chick blastula (unincubated blastoderm). NELSON SPRATT AND HERMANN HAAS.

Typical development (through 10± somite stages) occurs in almost all unincubated blastoderms explanted upper surface against a new yolk-albumen extract agar medium. This method has made possible for the first time an extensive analysis of the blastula stage (24–28 hours post-fertilization) in the freshly-laid chick egg.

The bilaterally symmetrical organization of the unincubated blastoderm, although quite evident in a cell density gradient and pattern of lower layer morphogenetic movements, is in no sense irrevocably determined either in its position or direction. Complete transection and longitudinal section, partial longitudinal section with a sizable connecting bridge of pellucid and opaque area cells, separation of the prospective posterior quadrant from the remaining three-fourths of the blastoderm, etc., in short, any cut through or into the system stimulates regenerative growth which tends to transform the outline of a fragment into a circle, *e.g.*, like that of the intact whole blastoderm. This in turn may bring about either: (1) a change in direction of the median plane of lower layer movement (and consequent position of the embryonic axis upon the blastoderm), (2) complete reversal in polarity of the movement pattern (and of the direction of the embryonic axis), (3) induction of one or two new secondary bilateral patterns of movements (one or two complete secondary embryos) or (4) division of the normal axial pattern into two bilaterally symmetrical movement patterns (two complete embryos).

The unincubated blastoderm is thus capable of great regulation and regeneration and consequently exhibits the properties of an embryonic field. Any part of the system large enough (exceeding about ¼ of the original mass) is able to form a complete, bilaterally symmetrical embryo morphologically and functionally equivalent to a normal 10± somite stage. About 80 twins and 7 triplets have been obtained to date.

Whole-mount preparations of fish lens epithelium from various species. B. Dobli Srinivasan.

Whole-mount preparations of epithelium from the crystalline lens of rabbit, rat and frog have proven useful in studies on cellular proliferation in normal and injured tissues (Harding *et al.*, 1960). The unique characteristics of the lens (*e.g.*, its avascularity, lack of a nerve supply, enclosure within a membrane, presence of epithelium as a single layer of cells) have been shown to be of advantage in studies on the relationship between injury, and DNA synthesis and mitosis in the rabbit lens (Harding *et al.*, 1959). It would be of interest, for a number of reasons, to extend this study to include the lenses of cold-blooded animals, and the present report indicates that whole-mounts of the entire layer of lens epithelium can be prepared from the following species: sea bass (*Centropristes striatus*), dogfish (*Mustelus canis*), dusky shark (*Carcharhinus obscurus*), goose fish (*Lophius americanus*), sea robin (*Prionotus carolinus*), tautog (*Tautoga onitis*), scup (*Stenotomus versicolor*), and skate (*Raja erinacea*).

A procedure previously described (Harding, 1960) was followed in making the whole-mounts. In all cases essentially complete layers of epithelium could be prepared. They consisted of a single layer of cells. Stainability with Harris' hematoxylin varied from species to species. Good results were obtained with dogfish, dusky shark, goose fish and skate. Mitotic figures were found in preparations from dogfish, sea bass, scup, tautog and skate. Mitotic figures, in these species, were found scattered over a relatively wide area as compared with the peripheral band seen in the rabbit and rat previously described. Lenses from goose fish and dusky shark were not fixed immediately after death of the animals and mitotic figures were not seen in these specimens.

It is concluded that the entire layer of lens epithelium, in the form of a whole-mount, can be prepared from a number of species of fish, and that a few of these species, namely dogfish and skate, appear to be very suitable for studies on cellular proliferation.

Extraction of chlorophyll from marine plankton algae with acetone and methanol. Carol Sterns.

Standard spectrophotometric methods for the quantitative determination of chlorophyll are based upon the specific absorption of pure acetone solutions of the pigment. This technique has been widely adapted in ecological research for the measurement of chlorophyll in natural plankton populations. There are many indications, however, that chlorophyll extraction of marine plankton algae with cold acetone as a solvent is far from complete. This is particularly true of certain species (*i.e.*, many of the green algae) with tough, resistant cell walls. Higher chlorophyll values are consistently obtained if the cells (millipore-filtered or centrifuged from suspension) are subjected to mechanical grinding or sonification. Neither of these latter techniques is practical for field analyses or for measurement of large numbers of samples.

On the other hand, hot methanol, long used for chlorophyll extraction of higher plants, appears to be considerably more effective than acetone for pigment extraction of marine phytoplankton. While some modification of the standard formulae for relating optical densities at various wave-lengths to concentrations of the different pigments may be necessary, it is noteworthy that the red absorption peaks of methanol and acetone solutions of chlorophyll (664 vs. 661 mμ) are nearly the same, and that relative absorption at the respective peaks is only about 3% higher for methanol than for acetone.

A comparison of the optical densities at 665 mμ of cold 90% acetone and hot methanol extracts (methanol hot initially, extraction for 24 hours in dark and refrigeration) of a variety of organisms gave the following results (shown as O.D. methanol/acetone): *Nannochloris atomus* (green) 1.96; *Synechococcus* sp. (bluegreen) 1.60; unidentified diatom 1.18; *Dunaliella euchlora* (green) 1.00; *Amphidinium carteri* (dinoflagellate) 1.00; *Pyramimonas* sp. (green) 0.94; natural plankton from Vineyard Sound, 1.29. This work was carried out as a part of the student training program of the Marine Ecology Course.

Further inquiry into the mechanisms of aqueous humor formation in dogfish. WILLIAM STONE, JR., DANA FELDSHUH, JUDITH LEINING AND R. F. DOOLITTLE.

The osmotic pressure of the aqueous humor of the smooth dogfish (*Mustelus canis*) is lower than the osmotic pressure of its blood plasma. The osmotic pressures of both plasma (960 milliosmoles) and aqueous humor (935) are higher than that of the environmental sea water. It has been suggested that water moves across the cornea from the sea, thereby diluting the aqueous humor with respect to the plasma from which it is derived. If this were so, differences in the osmotic pressure of the sea water bathing the cornea should be reflected in the degree of dilution of aqueous humor. Smooth dogfish were subjected to sea waters of varying tonicities for times ranging from one to six hours. When the osmolarity was lowered as much as 100 milliosmoles below normal, neither aqueous humor nor plasma exhibited any significant change. When the osmolarity was raised, the plasma and aqueous humor osmolarities increased in parallel, the difference between them remaining constant. Furthermore, when the formation of aqueous humor was arrested by intravenous administration of Diamox (acetazoleamide), the aqueous humor osmotic pressure increased, in the limit reaching that of the plasma. These phenomena were interpreted as meaning that the difference in the osmotic pressures of dogfish aqueous humor and its plasma is independent of any water entering the eye across the cornea. A preliminary investigation of the enzymatic profile of the dogfish ciliary body confirmed a high level of carbonic anhydrase, inhibition of which could be effected by both *in vivo* and *in vitro* Diamox administration. The Q_{O_2} of dogfish ciliary body is higher than that of its retina, when compared on a dry weight basis. In addition, the two tissues respond differently to the presence of substrate (glucose) during respiration studies, the ciliary body Q_{O_2} being higher with glucose added (3.08) than the ciliary body endogenous (1.86), whereas retina endogenous is higher (1.13) than when added glucose is present (0.95).

Separation and isolation of Spisula embryo cells. CORNELIUS F. STRITTMATTER AND PHILIPP STRITTMATTER.

A technique has been developed for separation and isolation in mass of individual cell types from embryos of *Spisula solidissima*, in order to permit studies on enzyme components of various cell types during early embryonic development. The technique involves dissociation of embryo cells in calcium-free medium and subsequent isolation of cell types of different sizes by density gradient centrifugation.

Spisula embryos are harvested at a selected stage of development, washed twice with cold 0.52 *M* NaCl–0.02 *M* Tris buffer, pH 8, (NaCl) to remove calcium, then suspended in cold 0.07 *M* sucrose–0.02 *M* Tris buffer, pH 8 (SM). On prolonged standing in this medium, the cells of the embryo dissociate slowly, but complete dissociation is rapidly achieved by adding *ca.* 0.0005 *M* Versene and and gently agitating the suspension. The separated cells retain a normal appearance and are capable of further cleavage.

The subsequent density gradient centrifugation may be illustrated by the isolation from 4-cell *Spisula* embryos of the large D cell and a mixture of the small A, B and C cells. The suspension of dissociated cells in sucrose medium is diluted with NaCl medium to give a

60 SM : 40 NaCl mixture. A 2-ml. aliquot of this mixture is layered over 10 ml. of 75 SM : 25 NaCl in a 15-ml. tube and centrifuged at *ca.* 70 *g* for three minutes. A 2-ml. fraction drawn off from the gradient boundary contains an 80–90% pure mixture of A, B and C cells in *ca.* 20% yield, while a 2-ml. fraction from near the bottom of the tube contains 90% D cells in *ca.* 20% yield. The purity of each fraction may be increased by a second centrifugation.

The density gradient centrifugation is applicable to isolation of cell types from dissociated embryos of other developmental stages by appropriate modifications of medium densities and of the duration and force of centrifugation.

Electron transport in eggs, developing embryos and adult tissues of Spisula solidissima. Cornelius F. Strittmatter, Philipp Strittmatter and Carolyn Burdick.

Enzymes of electron transport in unfertilized eggs and in adult heart and liver of *Spisula solidissima* have been studied by spectrophotometric assay of homogenates and fractions obtained by differential centrifugation. Unfertilized eggs showed a high activity of DPNH-cytochrome *c* reductase (10-12 μmoles electrons/min./ml. eggs), DPNH-ferricyanide reduction and cytochrome oxidase (2–4 μmoles electrons/min./ml. eggs), appreciable activities of succinic dehydrogenase (0.13–.20 μmoles electrons/min./ml. eggs) and TPNH-cytochrome *c* reductase, and a lower DPNH oxidase activity. Adult tissues showed somewhat similar but distinctive patterns. Unfertilized eggs, heart and liver each contained a particulate fraction resembling mammalian mitochondria in (1) centrifugal behavior, (2) absorption spectra, which indicated the presence of cytochromes *a* (and a_3), *b* and *c*, (3) oxidative activities, including essentially the total cell content of cytochrome oxidase and antimycin A-sensitive succinic dehydrogenase and a large portion of the DPNH-cytochrome *c* reductase activity, and (4) staining reactions, being the major site of cyanide-sensitive Nadi and Janus green reactions.

Tests on homogenates from fertilized eggs and embryonic stages up to 22-hour swimming forms showed no marked changes from unfertilized eggs in cytochrome oxidase, DPNH-cytochrome *c* reductase and DPNH-ferricyanide reduction. As these activities were in excess of values reported in the literature for oxygen consumption of *Spisula* eggs and early embryos, it appears that these terminal electron transport capacities are not limiting factors involved in the reported increase of oxygen consumption rate during early stages of *Spisula* development.

The ratio of succinic dehydrogenase to cytochrome oxidase activity was markedly higher in adult heart than in unfertilized eggs or early embryos. This change may possibly reflect a differentiation of mitochondria.

Cardiovascular and respiratory activity in dog fish. Frederick N. Sudak and Charles G. Wilber.

Squalus acanthias and *Mustelus canis* were pithed anteriorly to the mesencephalic region and posteriorly from the 14th to 18th vertebrae. Blood pressures were measured with pressure transducers. Respiratory activity was recorded directly from the branchial muscles. Electrocardiograms were recorded using bipolar electrodes. Deep body temperatures were maintained at 23.0° C. ± 0.5° C. Average heart rates at this temperature were 46.8 beats/minute in *Mustelus*, 28.5 beats/minute in *Squalus*. The heart rate Q_{10} (23° C. to 33° C.) in *Mustelus* was 3. EKG's in *Mustelus* showed a P-R interval of 0.25 sec., QRS of 0.07 sec. and QT interval of 0.58 sec. The ST segment was isoelectric and the T wave biphasic. Cardiac rhythmicity bore a phasic relationship to respiratory activity in both species. RR intervals were constant when the ratio between respiration rate and heart rate was 1 in *Mustelus* and 2 in *Squalus*. Arrhythmias of various degrees were observed whenever the two cycles fell out of phase. Respiratory activity occurring during the middle third of a given cardiac cycle consistently prolonged the diastolic period of that cycle. Respiration rate in *Mustelus* was 48.7/minute, in *Squalus* 69.0/minute.

The average mean perfusion pressure from the ventral aorta to the venous side of the circulation was 22.4 mm. Hg. End diastolic intraventricular pressure varied from minus 0.7 to 0 mm. Hg with the pericardium intact. Average blood pressures in the ventral aorta were:

systolic 29.5 mm. Hg, diastolic 21.2 mm. Hg and pulse pressure 8.2 mm. Hg. The mean blood pressure showed periodic variations, resembling Meyer waves described in mammals. The periodicity was 2.8 cycles/minute in *Mustelus* and 0.8 cycles/minute in *Squalus*. The magnitude of the pressure change was 1.4 mm. Hg.

Supported in part by NIH grants H-3563 and MY-3235.

Cardiovascular responses to hemorrhage in the dog fish. FREDERICK N. SUDAK AND CHARLES G. WILBER.

Cardiovascular and respiratory responses to acute hemorrhage were studied in *Mustelus canis* and *Squalus acanthias*. All animals were pithed anterior to the mesencephalon and posteriorly from the 14th to 16th vertebrae. Blood pressures were recorded directly from the ventral aorta. Electrograms obtained from the branchial muscles were used to record respiratory activity. Blood pressures and respiration were recorded before, during and after the removal of blood from the caudal vein, and following reinfusions of blood into the ventral aorta. Systolic and diastolic pressures decreased linearly with the increase in blood deficit. Systolic blood pressure decreased an average of 1.8 mm. Hg/ml. of blood lost, while the diastolic pressure decreased an average of 1.0 mm. Hg/ml. of blood lost. A similar pressure-volume relationship was obtained when blood was reinfused. Mean blood pressures were reduced approximately 50% after removal of 5–7 ml. of blood (10% of estimated total blood volume) from a 700-gram fish. Changes in heart rate and respiration rate were inconsistent. In general, the cardiovascular system in both species behaved like a simple pressure-volume system in response to blood loss. No evidence was found to indicate the presence of a compensatory mechanism against decreases in blood pressure resulting from reduction of blood volume.

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Uptake and release of calcium-45 by Fucus vesiculosus. ELIJAH SWIFT AND W. ROWLAND TAYLOR.

Five to seven grams (dry weight) of whole young thalli of *Fucus vesiculosus*, without macroscopic epiphytes, were incubated in sea water in 260-ml. bottles maintained at 22° C. and under constant illumination (700 to 1300 foot candles, depending on the experiment). The bottles contained about 1 μC. of $Ca^{45}\,Cl_2$. The uptake or release of isotope was followed by removing aliquots of sea water, plating in the usual manner, and assaying in a gas flow counter.

The algae appeared to take up radiocalcium rapidly, most of the uptake occurring in the first hour. The isotope did not appear to be concentrated since calculations from Vinagradov's tables indicate the amount of isotope taken up was of the order of magnitude that would be expected if the radiocalcium in the sea water came into equilibrium with the calcium in the algae.

Fucus vesiculosus maintained in sea water containing calcium-45 was rinsed, and placed in millipore-filtered sea water to which no isotope had been added. In 24-hour experiments, 90% of the radiocalcium appearing in the medium was released in the first two hours. Similar studies were done with thalli that had been used in uptake experiments. If it is assumed that the radiocalcium in the thalli came into equilibrium with the medium in both the uptake and release experiments, the calculated and observed values check within 10%. These techniques did not show a significant difference between *Fucus* maintained in the dark or illuminated, in either the uptake or release experiments. These data indicate there is a rapid turnover of calcium by *Fucus vesiculosus*. (Partly supported by contracts between the Chesapeake Bay Institute, The Johns Hopkins University and the A.E.C. and O.N.R.)

The effect of photosynthesis by benthic macroalgae on the titration alkalinity of sea water. W. ROWLAND TAYLOR.

Six species of benthic macroalgae have been examined to determine the effect of their photosynthetic and respiratory activity on the alkalinity and pH of the sea water medium. The algae, 0.5 to 1 gram wet weight depending on species, were incubated in series of 125-ml.

light and dark bottles at constant temperature (21° C.) and illumination (1800 foot candles). Changes in the titration alkalinity and pH were noted and rates of photosynthesis and respiration were followed by oxygen measurements.

When *Ulva lactuca* or *Enteromorpha intestinalis* was incubated in this manner, the pH of the medium increased from 8.0 to 9.0 in about six hours but the alkalinity did not change. On continued incubation, the pH rose to 10.0 and the titration alkalinity decreased from initial values of 2.20 to 1.50 milliequivalents/liter. Oxygen determinations indicated continued photosynthesis at these high pH levels. Photosynthesis by *Ceramium rubrum, Chondrus crispus, Chorda filum* and *Fucus vesiculosus* resulted in an increase in pH to maximum values of 9.0–9.2. With one exception, there was no change in the alkalinity. In the case of the *Fucus* there was a small decrease in the alkalinity during the latter part of extended runs. Respiration by all of these algae resulted in a decrease in pH but no change in alkalinity. Preliminary experiments, using sea water in which the partial pressure of CO_2 had been lowered, indicated the rate of oxygen production by the *Fucus* and *Enteromorpha* species may be dependent on the concentration of dissolved CO_2.

These findings suggest that these algae probably use dissolved CO_2 as their carbon source under their usual environmental conditions. However, the two Chlorophyta can use bicarbonate (or carbonate) when growing at high pH. (Partially supported by contracts between The Johns Hopkins University and the A.E.C. and O.N.R.)

Uptake of iron-59 by marine benthic algae. W. Roland Taylor and Eugene P. Odum.

Exploratory experiments have been undertaken to investigate the uptake of iron-59 by marine benthic algae. A typical experiment consisted of placing a weighed amount of an alga in each of a series of stoppered bottles or flasks filled with sea water. Suitable amounts of $Fe^{59}Cl_3$ were added to the containers; they were maintained at constant temperature and either illuminated or darkened as required. The uptake of the isotope was followed by removing aliquots of the water and counting with a scintillation detector. In some experiments, pH and oxygen production were also measured. Such studies were complicated by adsorption of the isotope on the walls of the container, and it was necessary to apply corrections obtained from blanks or add chelating agents to the medium.

The isotope was taken up by *Ceramium rubrum* and *Enteromorpha intestinalis* rapidly and there was little loss of the isotope when algae were removed from the radioactive medium and placed in fresh sea water. A rough estimate of the concentration factor would be about 100 to 300. Addition of 30 mg./liter of the chelating agent ethylenediaminetetraacetic acid was found to prevent the loss to the surface of the container. In experiments lasting three to four hours, both the amount and rate of uptake was less in the presence of EDTA. There appeared to be no appreciable difference of uptake in light or dark bottles. In the case of *Ceramium,* addition of buffer ("Tris") to minimize the pH changes resulting from photosynthesis indicated pH does not effect the uptake.

In an experiment using *Fucus vesiculosus* it was found that the radioactivity in the medium decreased at a rate less than that in a blank containing no algae. It is speculated that the *Fucus* may release a chelating substance into the medium. (Partially supported by contracts with the A.E.C. and O.N.R.)

Nuclear division in Holosticha lacazei Maupas. Reuben Torch.

Holosticha lacazei, a relatively obscure, multinucleate, free-living, hypotrichous, marine ciliate, was collected on June 23 at Woods Hole and has been cultured in the laboratory for two months.

Nuclear division was studied in animals fixed in Champy's fluid, followed by bleaching in either 3% hydrogen peroxide or dioxan, and stained by the Feulgen reaction or with Heidenhain's hematoxylin. Champy's fixative was used since the animals dissolved in all fixatives except those containing osmium tetroxide.

The number of macronuclei in non-dividing individuals varies from 32 to 71, with most individuals having 40–50. The spherical macronuclei measure 4–8 μ and contain a central chromatin mass surrounded by numerous small, achromatic spheres. The homogeneous Feulgen-positive spherical micronuclei measure 2 μ and number from 5–13 in non-dividing individuals. There is no apparent correlation between the numbers of macronuclei and micronuclei.

Prior to nuclear division, the macronuclei fuse into an elongate, intensely Feulgen-positive mass in the center of the cell along the longitudinal axis. Concurrently, the daughter adoral membranellae are formed and the future division plane is demarcated. The micronuclei cluster around the fused macronucleus and proceed to divide. Micronuclear division is not synchronized, since no more than half the micronuclei divide at one time. Division of the micronuclei was not observed at any other stage in the life cycle. The micronuclei are distributed more or less equally among the daughters, but in one exceptional case, one daughter contained 6 micronuclei while its sister contained 15.

The macronuclear mass divides approximately in half, one product going to each daughter. Macronuclear divisions continue until each daughter contains 20–30 macronuclei, whereupon the daughters separate. The earlier macronuclear divisions are generally synchronized, but later divisions are sporadic. After separation of the daughters, many of the macronuclei continue to divide. Macronuclear reorganization was not observed at any stage in the life cycle.

Ammonia in Golfingia gouldii and ambient air. DAVID M. TRAVIS.

This work was begun with determination of normal levels of ammonia in ambient air and in coelomic fluid of *Goldfingia gouldii,* in order to devise approaches to a study of effects of increased environmental ammonia gas concentration on metabolic processes. For analysis of air, a large volume, greater than 400 liters, was passed through a cotton filter and two acid traps in series, in order to collect the ammonia. Coelomic fluid was analyzed directly and by use of a trichloracetic acid filtrate prepared immediately after withdrawal from the body cavity. Ammonia was determined by microdiffusion and colorimetric measurement of the reaction with ninhydrin-hydrindantin reagent.

Results were as follows: (1) The ammonia fraction in ambient air was 1.74×10^{-8} (the mean of 14 determinations ranging from 0.55 to 3.47×10^{-8}). (2) Total ammonia of coelomic fluid, using trichloracetic acid filtrate, was 230 micromoles per liter (the mean of 18 determinations, ranging from 115 to 560 micromoles per liter). (3) Direct determinations of ammonia on coelomic fluid at timed intervals after fluid was drawn from the animal showed a rising curve, the flat portion of which was used for extrapolation back to zero time. Values obtained by this extrapolation were about 25% lower than those obtained on the same specimen using trichloracetic acid filtrates.

The results of this work have led to the following approaches to further study of ammonia in physiologic processes: (1) large volumes of ammonia gas may be prepared, analyzed, and used in experimental situations; (2) serial samples of 1 ml. of coelomic fluid may be determined for ammonia in triplicate; (3) release of ammonia into the closed space, containing the animal and suitable reagents, of a respiratory flask may allow precise measurement of metabolic processes.

Respiratory changes induced in Golfingia gouldii by alteration in environmental carbon dioxide. DAVID M. TRAVIS.

Respiration of *Golfingia gouldii* at environmental carbon dioxide concentrations below and above approximately normal levels was studied in order to learn whether this gas exerts effects on the rate of oxygen consumption in the whole animal. For essentially zero carbon dioxide in surrounding air, a volumetric respirometer was used in which carbon dioxide was removed by alkali. For normally small and larger concentrations of environmental carbon dioxide, a syringe method was adopted which allowed measurement of changes in oxygen concentration of a known volume of gas from which oxygen consumption could be calculated. Actual oxygen concentrations, kept between 19.7 and 20%, changed less than 0.5% during periods of one hour.

At zero carbon dioxide tension, oxygen was consumed by the animal at a rate of 33 microliters/hour and gram of wet weight to the 2/3 power ($W^{2/3}$) (mean value for 13 observations on 4 worms, ranging from 20–53). At low concentrations of 0.36–0.99% carbon dioxide, obtained by allowing equilibration for one hour with room air, the rate was 45% lower, or 15 microliters/$W^{2/3}$-hour (mean of 9 observations on 6 worms, range 9–19). In simultaneous experiments using pairs of worms crossed alternately between low concentrations of 0.49, 0.91 and 0.48% carbon dioxide and higher concentrations, the oxygen consumption was reduced by 27, 35 and 70% when the concentration was raised by 1.0, 3.5 and 8.0% above the lower levels. Oxygen concentrations were approximately the same at the two levels.

From these results it is suggested that carbon dioxide depresses aerobic respiration in *Golfingia gouldii* in approximately physiologic as well as higher pharmacologic concentrations, and, indeed, appears to accelerate respiration when removed from the environment. Studies of mechanisms involved and of the actual role carbon dioxide may play in natural surroundings are indicated.

Studies of cannibal giant Blepharisma undulans. N. TULCHIN AND H. I. HIRSHFIELD.

Previous studies have shown that selected feeding will induce formation of cannibal giant types of the pink, heterotrichous, fresh-water ciliate, *Blepharisma*. The ingestion of smaller blepharisma by the giant forms occurs readily. The fate of the ingested forms can be easily followed in the food vacuole. The digestion of the ingested forms and egestion of non-absorbed substances occurs within twelve hours.

Feulgen and methyl green-pyronin preparations show the DNA and RNA of the cannibal and of the ingested forms within the food vacuole. During digestion, the DNA in the food vacuole is progressively hydrolyzed, as shown by decreasing affinity for methyl green and Feulgen stains. However, pyroninophilia of the mass within the vacuole fluctuates during digestion. RNA-ase and perchloric acid treatments indicate that RNA may persist or accumulate in the vacuole during digestion and that some is present in the egested residues.

The cannibal giant forms have appreciably larger amounts of macronuclear DNA than bacteria-fed controls. The large quantities of DNA in the food vacuoles of the cannibals may thus contribute to the increased polyploidy of the cannibal giant forms.

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Differential staining of cytoplasmic inclusions in eggs of Pectinaria. KENYON S. TWEEDELL.

Constituents of the egg cytoplasm were studied in the germinal vesicle stage, during germinal vesicle breakdown and in the primary oocyte. Inclusions of living eggs were observed before and after centrifugation, using vital fluorochromes, other specific vital dyes and dark-field microscopy. In addition, cytochemical studies were made on fixed eggs before and after centrifugation.

Besides germinal vesicle, nucleolus, chromosomes and spindle, many highly motile inclusions were observed vitally. The latter included yolk, mitochondria, astral granules, germinal vesicle substance, oil droplets and chromidia. Additional stationary, peripherally located granules were seen. By specific cytochemical staining reactions (vital and non-vital) these inclusions could be further differentiated.

Of the vital fluorochromes utilized, acriflavine, thioflavine and acridine orange gave polychromatic staining. Others dyed specific particulates. The fluorescent cell components include cell and nuclear membranes, nucleus, nucleolus, yolk, mitochondria and other unidentified granules.

Two of the vital dyes, toluidine blue and nile blue sulphate, stain several cell components in the germinal vesicle stage. These include nucleolus, peripheral cell granules, yolk and a heavy zone of granules surrounding the germinal vesicle. Both dyes stain the yolk particles blue and show metachromasia with other granules. After centrifugation the metachromatic granules become stratified into separate bands. With toluidine blue, a thin red-violet band of particles occurs just beneath a large cap of unstained granules and droplets at the centripetal pole. When nile blue sulphate is applied, a different red-violet band of small particles stratifies out, just centripetal to the yolk. The astral granules, which become associated with the asters of the first maturation spindle, are derived from this latter band.

Reduction and delay of cleavage in Chaetopterus eggs by acridine orange. K. S. Tweedell and C. D. Watters.

Recently, acridine orange was shown to have an inhibitory action on mitosis in *Arbacia* (Rustad, 1959). Additional effects on fertilization and early cleavages were observed when acridine orange was utilized as a vital fluorochrome to follow early development in *Chaetopterus*. The staining period, concentrations of A.O. in sea water, choice of gametes and time of application all affect the number of eggs cleaved.

Acridine orange was applied in concentrations of 10^{-3} (15 minutes), 10^{-4} (30 minutes) and 10^{-5} (60 minutes) in each experiment. When sperm were stained prior to fertilization, the percentage of eggs reaching first cleavage increased (72 to 81%) as the stain intensity decreased. Previously stained eggs in the same concentrations showed an increase in cleavage from 50 to 60% as stain concentration decreased. When both sperm and eggs were dyed prior to fertilization, the percentage of eggs cleaved did not vary significantly from eggs stained alone. Average cleavages in controls for all groups remained constant between 80 and 82%. This suggests that the inhibitory effect on cleavage was principally mediated through the egg.

When eggs and sperm were fertilized in 10^{-3} A.O. and removed after 15 minutes, cleavages were sharply (10%) curtailed. In 10^{-4} A.O., 27% of the eggs cleaved but in 10^{-5} A.O. cleavage recovered (84%). This suggests the factors here either act during fertilization or affect some phase of the cleavage process.

Application of the threshold concentration (10^{-5}) to the egg alone also caused a slight delay (3 minutes) in the first cleavage. When sperm or sperm and eggs are dyed prior to fertilization, an identical delay of 10 minutes occurs. This suggests a sperm-induced cleavage delay. In each case, the rate of development remained constant with the controls. Eggs and sperm fertilized in the stain (then removed) showed no delay in first cleavage, but the rate of succeeding cleavages decreased.

Phosphagen and nucleotides of Limulus muscle. G. W. de Villafranca, J. E. Ross and A. M. Morgan.

Horseshoe crab muscle was extracted with trichloracetic acid and precipitated as the alcohol-insoluble barium salt. It was hydrolyzed and the free base chromatographed with arginine, taurocyamine, glycocyamine and creatine standards. The average R_f values in 3 solvents (propanol-ammonia-water, butanol-acetic acid-water, and butanol-ethanol-water) were: arginine, 0.43, 0.14 and 0.07, respectively, and closest to the values found for the *Limulus* base of 0.43, 0.13 and 0.07. The amount of phosphagen, arginine phosphate, was determined by colorimetric assay of the total arginine and the free arginine, the difference being due to bound arginine and assumed to be arginine phosphate. The average values for 6 different animals were 6.26 mM of arginine and 3.10 mM of bound arginine per 100 grams of muscle, wet weight. It is of some interest that the values were always greater than zero, and relatively constant, once it was realized that the crabs must be used fresh. The amount of AP probably decreases once the animal has been removed from its natural habitat; it apparently decreases rapidly and to zero in the running sea water tables.

The nucleotide composition was determined by ion-exchange chromatography (Dowex in the formate cycle) on both barium salts obtained by TCA extraction and, later, on neutralized perchloric acid extracts. In the former case, ATP was the major component with an average of about 100 μM per 100 grams of muscle, fresh weight. ATP was recovered from perchloric acid extracts in concentrations ranging from 163 μM to 338 μM, with an average of 265 μM per 100 grams of muscle. Traces of ADP (43 μM), AMP (26 μM) and other, as yet, unidentified, minor nucleotides were also found.

Work supported by USPHS grant A-2647 and an ONR grant administered by the M.B.L.

Oxygen consumption in Uca pugnax: seasonal variations. H. Marguerite Webb.

O_2-consumption of the fiddler crab, *Uca pugnax*, was recorded continuously from July 25, 1959, through October 6 and for six semi-monthly periods between October 11, 1959, and February 18, 1960; a further lunar period was recorded from June 25 to July 23, 1960. The mean hourly rate of O_2-consumption was found to be highest during the months of July and August, and to be lowest (about 55% of summer values) during the period from November to February. No gross changes in the form of the *diurnal* rhythm were observed during the period of the investigation nor were there any consistent variations in the amplitude, which ranged from 28% to 43% of the mean hourly rate. When the data were analyzed for *lunar-day* rhythms it was seen that from July 25 through October 25 the rhythm exhibited a symmetrically bimodal form with maxima at lunar zenith and nadir. During the period from November 19 through January 23, although the lunar-day rhythm was less regular than for the earlier periods, it was clearly uni-modal with a maximum about an hour after lunar zenith. During the period from February 2 to 18 the lunar rhythm resumed its bimodal character, but with minima at lunar zenith and nadir. It thus appeared to be inverted with respect to the "summer" rhythm. Like the diurnal rhythm, the lunar rhythm exhibited no systematic changes in percentage amplitude throughout the period studied. During the period from June 25 to July 23, 1960, the lunar rhythm was found to have the "summer" form and phase relations—bimodal, with maxima at lunar zenith and nadir.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

Effects of environmental changes on indefinitely prolonged action potentials of lobster muscle fibers. R. Werman, J. P. Reuben and H. Grundfest.

Prolonged spikes evoked in muscle fibers treated with a combination of procaine and some other agent (Reuben and Grundfest, this issue) were affected very little by drastic changes in the environment, made during the plateau of the response. The stability of the membrane potential and conductance during the plateau were particularly marked with the responses that had a high membrane conductance (Reuben, Werman and Grundfest, this issue). These spikes were not affected by replacement of all the ions in the bathing medium with sucrose or urea; by replacement of sodium with alkali earth or organic cations; by 20-fold increase in potassium; by substitution of chloride with various anions; or by maximal activation of the inhibitory synapses with γ-aminobutyric acid (GABA).

The spikes with lower-conductance plateaus could be affected to some degree. Bathing the fibers in sucrose or urea induced oscillations, between the plateau and more negative values. Substitution of SCN ions for chloride, which hyperpolarized and increased the membrane resistance of resting muscle fibers, abolished the spikes. Occasionally, the addition of GABA also abolished the spikes.

These findings suggest that in its "upper stable state" the membrane does not behave as a simple electrode for ionic changes. This is particularly the case for conditions in which the "upper stable state" is associated with high membrane conductance.

Relative effectiveness of inhibitory membrane in different fibers of a lobster muscle. R. Werman, J. P. Reuben and H. Grundfest.

During its maximum activity, the inhibitory membrane of the fibers operates as a high conductance Cl^--electrode, tending to set the membrane at the Cl^--equilibrium potential. The degree to which the synaptic activity prevents changes in the membrane potential that are normally produced by various means is a measure of the relative area of the synaptic membrane in different fibers.

The inhibitory synapses were maximally activated with GABA, in Ringer's solutions containing various amounts of K^+, or none. In the absence of GABA, the membrane potential changed nearly linearly with log K^+, with a slope of 54 mV/decade change in K^+. In zero K^+ there was hyperpolarization of 10 to 15 mV. When the synaptic membrane was activated by GABA, the membrane potential changed relatively little on removal of K^+ in most fibers and not at all in many. On raising K^+ above 15 meq./l. (the value in *Homarus* saline) the slope of the change was again 54 mV/decade change in K^+, but with a different origin. Removal of K^+ during tetanic activation of the inhibitory synapses also produced less hyperpolarization, but abolition of all hyperpolarization, as with GABA, was not seen.

The variation of the clamping action of inhibitory synaptic activity indicates that the conductance of the synaptic membrane was less in some fibers than in others, presumably because different fibers have different areas of innervated membrane. Block of propagation is readily produced during high frequency activity of the inhibitory axon, and accounts for the lower effectiveness of synaptic clamping as compared with that of GABA. The identical slopes of the depolarization curves in high K^+, with and without GABA, reflect the increased conductance of depolarized membrane, and they provide further evidence that the activity of the inhibitory membrane does not involve K^+-conductance.

In situ measurements of the community metabolism of Quohog Pond. George C. Whitely, Jr.

In situ estimates of oxygen production in Quohog Pond, West Falmouth, were obtained by means of a polarographic oxygen electrode during September, 1959. In July, 1960, the water column at one station was analyzed by Winkler technique for dissolved oxygen every three hours for a duration of 24 hours. Rates of respiration, diffusion, and total photosynthesis were computed graphically for both the September and July data.

Total photosynthesis in a water column one square meter in area was 7 grams oxygen/m^2/day or 2.9 grams carbon/m^2/day for September, 1959; 24 grams oxygen/m^2/day or 10 grams carbon/m^2/day for July, 1960.

During June, 1960, the Marine Ecology Course class study of Quohog Pond determined primary production of the phytoplankton *in vitro* (light and dark bottle method) to be 0.5 gram carbon; benthic algae by the same method averaged 10 grams carbon. Low value for the phytoplankton suggests that bottle measurements alone may have some of the same disadvantages in Quohog Pond as have been found elsewhere; *i.e.*, shallow Texas bays, where such measurements do not take into account the metabolism of benthic organisms.

Production of viable races of Paramecium caudatum after micronuclear elimination with x-rays. Ralph Wichterman.

X-irradiation has been shown to affect micronuclear number in several species of ciliate Protozoa. As a result, there is a diminution of the number of micronuclei and, with reasonably high or repeated dosages, their complete disappearance.

Specimens from clonal cultures of opposite mating types of *Paramecium caudatum* (9 and 10 of variety 5) were treated as follows: 200 counted paramecia were placed in each of four Nylon syringes (2-ml.) and irradiated simultaneously with dosages of 150, 200, or 250 kr, all of which were below the LD 50 value. Immediately after irradiation, some specimens were examined and the remainder expressed from the syringes into flasks of lettuce medium which contained *Aerobacter aerogenes* as the food source. Upon regaining reproductive ability, progeny of x-rayed survivors were then harvested, placed again in syringes and irradiated as before, while others were killed and stained for further cytological study. In this manner seven, approximately evenly spaced irradiation exposures were given over a 52-day period, to bring the cumulative clonal dosage to 1100 kr.

When specimens of opposite mating type from cultures which had received the cumulative clonal dosage of 1100 kr regained reproductive ability and later were mixed, mating and conjugation followed.

Paramecium caudatum has but one compact micronucleus, measuring approximately 8 μ. Repeated and successive x-irradiation of paramecia and their progeny in the manner reported here results in a gradual disappearance of the micronucleus or the production of a "ghost-like" condition of this structure as revealed in stained preparations, whereas the micronucleus from unirradiated controls stains intensely. At the highest cumulative clonal dosage, dividing specimens show either no micronuclear spindle (since such paramecia are now amicronucleate), or else a dividing "ghost-like" micronuclear spindle in contrast to the conspicuous, dividing micronucleus of the unirradiated controls. At least in appearance, the structure of the macronucleus appears little affected by the irradiation.

Part of a project aided by a grant from the Committee on Research, Temple University, and a contract between the Office of Naval Research, Department of the Navy and Temple University (NR 104-475).

The effect of LSD 25 on heart rate in Cistenides. CHARLES G. WILBER.

In previous work, it was shown that the pulsation of the dorsal blood vessel in the trumpet worm is temperature-dependent. The rate response to temperature may be described as $y = 3.5\,x - 49$, where y is beats per minute and x is degrees Centigrade. Approximately 50 worms were immersed in different concentrations of lysergic acid diethylamide (LSD 25) in sea water at 22° C. The effect of this treatment on pulsation of the dorsal vessel was recorded. By 30 minutes after immersion in 20 micrograms LSD 25 per ml. sea water, the rate of pulsation of the dorsal vessel was increased to 130% of control. In more concentrated solutions, the worms became flaccid, extended, and the dorsal vessel ceased to beat. In sea water containing 10 micrograms per ml. LSD 25, there was no change in rate, or possibly a depression to 10% of control. By way of comparison, worms immersed for 30 minutes in 10 micrograms per ml. atropine sulfate in sea water showed a decrease to 59% of control rate. In 20 micrograms per ml. the rate increased to 120% of control. After two hours in the above solutions, the rates were essentially the same as those indicated above. The LSD 25 was kindly supplied by the Sandoz Pharmaceutical Company.

Supported in part by National Science Foundation Grant 4005.

Cardiovascular and respiratory reflexes in elasmobranchs. CHARLES G. WILBER AND FREDERICK N. SUDAK.

During a simulated dive, certain mammals, birds and reptiles show a profound bradycardia with accompanying changes in blood pressure. Fish are said to show similar changes if removed from the water. To ascertain in some detail the nature of such responses in elasmobranchs, we used specimens of *Squalus acanthias* and of *Mustelus canis*. The unanesthetized fish were immobilized in a plastic trough. Fresh sea water was run into the mouth through a plastic hose. Blood pressure was recorded directly from the ventral aorta with a pressure transducer. Heart rate was read from the pressure record. Respiration was recorded through electrodes placed directly into the branchial muscles. Control records were made with full water flow. The water was then shut off; heart rate, blood pressure and respiratory rate were monitored for several minutes. Within 6 to 20 seconds after water flow was stopped, the following physiological responses (expressed as change to per cent of control) occurred: *Squalus* heart rate 45%, respiratory rate 51%, pulse pressure 144%, mean pressure 82%; *Mustelus* heart rate 42%, respiratory rate 19%, pulse pressure 214%, mean pressure 90%. The heart rate response in both species was abolished by atropinization of the fish. Atropine abolished the pulse pressure response in *Squalus* but not in *Mustelus*. The observed bradycardia is apparently mediated through the vagus nerve. Atropinization did not modify the respiratory response in either species. The small changes in mean blood pressure, despite relatively large decreases in heart rate, after interruption of water flow, do not confirm the view that this response serves to protect delicate gill structures.

Supported in part by research grants MY-3235 and H-3563 from the National Institutes of Health.

Some effects of lysergic acid diethylamide on circulation in elasmobranchs. CHARLES G. WILBER AND FREDERICK N. SUDAK.

Lysergic acid diethylamide (LSD 25), a psychotogenic agent in man, is known to provoke bradycardia in mammals by central vagal stimulation. The agent induces a drop in blood pressure as a result of depression of the vasomotor center, despite the vasoconstrictive action which the drug exhibits in perfused blood vessels or in spinal animals. We made some experiments with LSD 25 on *Mustelus canis*. Blood pressure and heart rate were measured before and at different times after various doses of LSD 25 were administered via the ventral aorta. Doses from 0.01 to 1.3 micromol per kg. body weight were given. After all doses there was a rise in systolic, diastolic, mean, and pulse pressures. Heart rate, which decreased by 40% at a dose of 1.3 micromol per kg., showed no decrease at doses less than 0.01 micromol. The rise in mean pressure amounted to 15% of control value at a dose of 0.01 micromol; 45% at 0.06 micromol; 81% at 0.56 micromol. Various methods of plotting dose against blood pressure response were tried. At the moment, the data seem to fall along a curve in the most orderly fashion if dose in micromol per kg. is plotted against the reciprocal of the per cent change in blood pressure. The results are now being processed through a computer to arrive at the best mathematical expression of the dose-response curve. The LSD 25 was kindly supplied by Sandoz Pharmaceutical Company.

Supported in part by research grants MY-3235 and H-3563 from the National Institutes of Health.

An autoradiographic study of the regenerating limb in the adult newt, Triturus viridescens. Ruth T. Wilf.

The source of cells for the blastema of the regenerating limb of the adult newt, *Triturus viridescens,* has been investigated, using autoradiography. In this two-year study the radioisotopes used were tritiated thymidine (principally), and C^{14} isotopes of glycine, leucine, and methionine. With tritiated thymidine, we assume takeup of radioisotope only in DNA-synthesizing, premitotic cells. In limbs with high epidermal cell labeling compared to control non-regenerating limbs, or with positive internal cells, regenerative activity of these cells is assumed.

With tritiated thymidine the approaches were: (1) time course regeneration study from third to twenty-eighth day post-amputation; injection and fixation on same day to give immediate picture of new cell formation; (2) newts injected from third to twenty-first day; fixation on subsequent days to study cellular divisions and migrations; (3) following the discovery that prior to the fifth day, only epidermal cells were labeled, tritiated thymidine was injected four hours pre-amputation or three days post-amputation, to test a possible epidermal cell contribution to blastema.

It was found that internal mesodermal components contribute to the blastema. From the fifth day many dedifferentiating muscle nuclei, muscle sheath nuclei, and some subcutaneous nuclei are synthesizing DNA. The periosteum is strikingly labeled. Schwann cells are generally negative. These conditions persist until after the blastema is well formed. The labeled dedifferentiated mesodermal cells divide and contribute extensively to the blastema. The blastema itself has a high rate of synthetic activity, which falls off as the blastema lengthens.

The epidermis is usually particularly heavily labeled in a band at the level of the amputation. These cells migrate distally continuously until about the eighteenth day, to form the bulk of the apical cap of epidermis, although some DNA synthesis is almost always taking place across the top of the cap. There is no evidence that epidermal cells contribute to the blastema.

Heat tolerance and thermoregulation in the fiddler crab, Uca pugilator. Jerrel L. Wilkens.

Fiddler crabs often feed in their natural habitat during the hottest part of a day in direct sunlight. The following experiments were undertaken to determine the upper extremes of temperature fiddler crabs can tolerate, and, under these conditions, if the effects of evaporation and radiation would be of any survival value. To determine environmental heat tolerances, freshly collected animals were subjected to temperature increases for one-hour periods over sea water and in dry air. In saturated air, 50% of the population died at 40.5° C. and 100% at 42° C. In dry air, 50% died at 45.5° C. and 100% at 47° C. The crabs, therefore, have a thermal tolerance of as much as 10 to 15° above average daily temperature (about 30° C.) for the Woods Hole area. However, temperatures approaching 45° C. were occasionally recorded on the surface of the sand; therefore, some thermoregulatory mechanism would be an advantage.

Through the use of thermocouples, internal temperatures of light-adapted and of dark-adapted specimens were determined during exposure to infra-red radiation in still air. The body temperatures approached equilibrium about 6° C. below ambient air temperatures in light crabs and 3° C. below air temperatures in dark crabs, for periods up to 15 minutes. These plateaus are due primarily to radiation and evaporation. To determine the effects of evaporation alone, crabs were subjected to slowly moving air at 37.5° C. Body temperatures leveled off at 33.5° C. in dry air, at 35.5° C. in air at 50% relative humidity, and at 37.5° C. in saturated air. The rate of evaporation of water was essentially proportional to increasing temperature.

This work was undertaken as part of the student training program of the Marine Ecology Course, and was partially supported by the Graduate School of Tulane University.

Experiments with ovulation induced in vitro by means of steroids in frogs and marine fishes. PAUL A. WRIGHT.

Certain androgenic, progestational, and cortical steroids have potent ovulatory capacity on isolated lobes of frog ovary, *Rana pipiens,* when added in amounts ranging from 2 to 0.001 mg. in 50 ml. of Ringer's solution and in the absence of pituitary factors. Several compounds (*e.g.,* progesterone, corticosterone, cortisone-21-alcohol) induced responses equaling or exceeding those brought about by optimal concentrations of frog pituitary extract. These same classes of steroids in moderate dosage enhanced ovarian response to pituitary factors *in vitro,* but overdoses often inhibited ovulatory responses. Estrone, estradiol, and diethylstilbestrol were incapable of inducing ovulation by themselves, and inhibited pituitary-induced ovulation, diethylstilbestrol > estradiol > estrone. Interestingly, these same estrogens did not inhibit ovulation *in vitro* induced by progesterone, testosterone, or hydrocortisone, but instead augmented the ovarian response to these ovulatory steroids.

Attempts to induce ovulation in excised ovaries of marine fishes (*Lophius, Fundulus, Tautoga*) by means of either steroids or pituitary extracts were not successful. A few eggs were released from a ripe ovary of *Fundulus majalis* with both progesterone and *Fundulus* pituitary extract, but controls in Ringer's solution also released ova spontaneously.

Aided by research grant A-2986 from the National Institutes of Health, USPHS. Thanks are due to Dr. Preston L. Perlman of Schering Corporation for supplying a variety of steroids for these experiments.

Metabolism of three species of the gastropod genus Littorina in and out of water. ROGER G. ZIEG.

Results of the experiments conducted on three intertidal snails of the Woods Hole region demonstrated that the respiration rate of these animals varied significantly when in water and when exposed. The three forms considered are dispersed, in more or less definite zones, on the shore. *Littorina palliata* is primarily in an aquatic habitat among the sea-weeds, *Littorina rudis* is confined to the upper regions of the shore exposed during low tide, *Littorina littorea* appears to be intermediate, being neither restricted to the exposed nor the submerged zones.

Rate of oxygen consumption was determined by Winkler method for measurements "in water," and a manometric method for measurements "out of water." Snails were collected at random and respiration measurements obtained for periods not exceeding three hours.

Results obtained by these methods, from about 500 snails of each species, indicate that: *L. palliata* gave mean values of 1.60 ml. O_2/gm./hr. in air and 1.01 ml. O_2/gm./hr. in water ($P < .001$). Results indicate that *L. littorea* consumes .54 ml. O_2/gm./hr. in air and .34 ml. O_2/gm./hr. in water ($P < .001$). On the other hand, *L. rudis* has lower respiration rates in air than in water, mean values of .59 ml. O_2/gm./hr. in air and .91 ml. O_2/gm./hr. in water ($P = .01$) being obtained.

The metabolic rates measured in air and in water tend to reflect the differential distribution of the three gastropods in the intertidal environment.

This work was carried out as a part of the student training program of the Marine Ecology Course.

A study of the nucleic acids of the dogfish cornea. SEYMOUR ZIGMAN AND WILLIAM P. AREND.

Our objective is to determine whether nucleic acids, due to their influence over protein synthesis, have a function in the maintenance of tissue transparency. Initial efforts were directed toward the development of a procedure for measuring the nucleic acid phosphorus content of smooth dogfish (*Mustelus canis*) corneas.

A modification of the Schmidt and Thannhauser method for the fractionation of acid-soluble, lipid, ribonucleic acid (RNA), deoxyribonucleic (DNA), and protein phosphorus components of the cornea was employed. All fractions contained appreciable amounts of phosphorus, both in the epithelium and stroma, except the protein phosphorus, which was nearly absent. As was expected, the highly cellular epithelium was far richer in RNA and DNA phosphorus than the very fibrous stroma.

These results led us to investigate the uptake of P^{32} by the compounds mentioned. Several incubations of whole corneas or of separated epithelia and stromas were attempted, incubating with elasmobranch Ringer's solutions containing 1 μc. of P^{32} for two and four hours. Since little incorporation of radioactivity into the nucleic acids was observed, it seems that more suitable systems must be sought.

Further work was carried out to develop a method for the quantitative analysis of the nucleotides of corneal RNA by paper electrophoresis. Although complete separation of 50 μg. of pure nucleotide solutions or of yeast RNA hydrolysates could be accomplished, corneal nucleic acid hydrolysates have not as yet given clear-cut separations of cytidylic, adenylic, guanylic and uridylic acids.

Future studies will include similar experiments on such transparent tissues as teleost corneas, avian nictitating membranes and squid integument.

Isolation of mitotic apparatus from pressurized Arbacia eggs. ARTHUR M. ZIMMERMAN AND DOUGLAS MARSLAND.

Mitotic apparatus (spindle-aster-chromosome complex) were isolated from the eggs of *Arbacia punctulata* immediately subsequent to a short exposure to high (up to 10,000 lbs./in.^{2}) pressure, applied at metaphase of first cleavage. The isolation procedure consisted of selective solubilization with digitonin after treatment with cold 35% ethanol.

In general it was found that high pressures had marked disorganizing effects upon the structure of the mitotic apparatus. However, the disorientation of the spindle-aster configuration decreased progressively as the magnitude and duration of the treatment were decreased.

A pressure of 10,000 lbs./in.^{2} applied for only one minute yielded spindle-aster remnants completely devoid of any detectable linear or radial structure, although the centriole areas and chromosomes were clearly visible. The chromosomes were clumped very irregularly near the center of the spindle area. Often the spindle area was abnormally short and sometimes curved.

In contrast to the foregoing result, seemingly normal spindle-aster complexes were recovered from eggs exposed to 4000 lbs./in.^{2} for one minute. The fibrillar structure of the spindle and asters was clear, the chromosomes and centriole areas appeared normal, and judging from the large number of anaphases found, movement of the chromosomes had not been abolished.

At intermediate pressure (8000 lbs./in.^{2}) a longer treatment (5 minutes) was required to approximate the effects previously described, and a still longer treatment (10 minutes) produced even greater disorganization.

Presumably the observed disorganization of the spindle and asters results from a pressure-induced solation of these intracellular gel structures. Moreover, the evidence suggests that the spindle is slightly more susceptible than the asters to such solation.

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Uptake of adenosine nucleotides in Arbacia eggs. ARTHUR M. ZIMMERMAN AND SELMA B. ZIMMERMAN.

Studies were conducted to determine the mode of penetration of adenosine triphosphate (ATP) and related adenine nucleotides into the eggs of *Arbacia punctulata.* Arbacia eggs were immersed into C^{14} labeled adenosine triphosphate, adenosine diphosphate and adenosine monophosphate, and subsequently prepared for autoradiographic analysis.

Experiments with unfertilized eggs immersed into C^{14}ATP (at 20–22° C.) for varying periods of time show that effective uptake was obtained after 3–4 hours immersion at relatively low ATP concentrations (5×10^{-4} to 10^{-3} M). Shorter immersion times of 60 and 120 minutes resulted in negligible amounts of C^{14} uptake. The uptake of labeled ATP in fertilized eggs was greater than that in unfertilized eggs. When eggs were placed into 5×10^{-4} M C^{14}ATP (0.12 μc/ml.) 5 minutes after insemination, an appreciable uptake of radioactivity was observed at the following metaphase. Qualitatively, the amount of uptake after 30–35 minutes incubation in the nucleotides was comparable to that found in unfertilized eggs incubated for a duration of 3–4 hours.

Preliminary observations of ATP-treated eggs (fertilized and unfertilized), centrifuged just prior to fixation, indicate that the radioactivity within the eggs is associated with the mitochondrial and yolk zones. Further studies are being conducted in order to determine the mechanism of uptake of ATP, ADP, and AMP.

This work was done with the technical assistance of Miss Patricia Rodgers.

Supported by grant G-6416 from the National Science Foundation.

A report of hermaphroditism in Arbacia punctulata. ARTHUR M. ZIMMERMAN, SELMA B. ZIMMERMAN AND ETHEL BROWNE HARVEY.

Hermaphroditism is not frequently found in the sea urchin, *Arbacia punctulata.* To date there have been only seven reported instances in this species. The hermaphrodite described in this report was given to the investigators at the Marine Biological Laboratory, Woods Hole, by Dr. E. E. Palincsar on the 17th of June, 1960.

Viable eggs and sperm were obtained simultaneously from the hermaphrodite upon electrical stimulation. Four of the gonads yielded eggs and the fifth gave sperm. Controlled electrical stimulation made it possible to collect unfertilized eggs. These eggs could then be fertilized with sperm from the same animal. Furthermore, cross-fertilization was possible, using sperm from another animal and eggs from the hermaphrodite. The unfertilized eggs averaged 71 μ in diameter. The fertilized eggs in the two- and four-cell stage measured 78 μ (without fertilization membrane). This is within the range of normal eggs.

Developmental studies under carefully controlled temperature conditions showed that the duration of time from insemination to 50% first cleavage furrows was 58–60 minutes at 20° C. This is similar to that found in normal control eggs. Both the cross-fertilized and the self-fertilized eggs were normal with respect to their morphology and rates of development to the pluteus stage.

The work was supported in part by grant G-6416 from the National Science Foundation and grant RG-7157 from the Division of General Medical Sciences, U. S. Public Health Service.

LALOR FELLOWSHIP REPORTS

On the natural inhibitors of glucuronosyl transferase present in serum of pregnant women. ROBERT M. DOWBEN.

The enzyme glucuronosyl transferase catalyzes the transfer of the glucuronide moiety from uridine diphosphoglucuronic acid (UDPGA) to a large number of hormones, metabolic products and drugs which are excreted as glucuronide conjugates. Previous studies disclosed the presence of inhibitors of this enzyme in the serum of pregnant women; one inhibitor was tentatively identified as pregnanediol-3α,20α, a metabolite of progesterone.

Ten liters of pooled serum obtained from pregnant women in the last trimester of pregnancy were acidified with sulfuric acid to pH 1.6 and extracted in batches with ethyl acetate for 12 hours by means of a continuous liquid-liquid extractor. All of the inhibitory activity of the serum was extracted by this means. The combined extracts were chromatographed on an alumina column, using a benzene-ethanol developer. A number of fractions possessing inhibitory activity have been obtained and assessed by measuring the decreased conjugation of phenolphthalein by a rat liver microsomal system in the presence of UPDGA. Aliquots of several of the more polar fractions lost most of their inhibitory activity after hydrolysis with β-glucuronidase. Similarly, the inhibitory activity of the pooled serum was greatly diminished by treatment with β-glucuronidase, indicating that some of the inhibitors may be glucuronides. The chemical identification of the various fractions is to be pursued.

The membrane surrounding milk fat globules. ROBERT M. DOWBEN AND DELBERT E. PHILPOTT.

The fat globules in cream were observed in the electron microscope. Fat in milk occurs as rather uniform globules about 3–4 microns in diameter. These fat globules do not coalesce to form butter unless the cream is churned, sonicated or frozen. Heat, treatment with fat solvents such as ethyl ether, and dilution with distilled water are much less effective in disrupting the fat globules. Calcium salts appear to stabilize the fat globules.

The cream of raw cow's milk was separated by centrifugation and washed three times with five volumes of 0.25 *M* sucrose containing 0.01 *M* $CaCl_2$. The third washings contained no detectable protein by the biuret reaction. Analysis of the washed cream revealed about 2.4 mg. protein/g. cream. The washed cream was examined in the electron microscope, using neutral mammalian isotonic osmic acid solution or neutral isotonic osmic acid dichromate solution for fixation.

The protein surrounding the fat globules appears to be a true membrane rather than adsorbed protein. The globules could be lysed by freezing and thawing, giving rise to discoid ghosts which resembled in many ways erythrocyte ghosts.

Serotonin (5-hydroxytryptamine) in the male reproductive tract of the spiny dogfish. THADDEUS MANN.

The male copulatory organs or "claspers" of the spiny dogfish, *Squalus acanthias,* by means of which semen is transferred from the male to the female, are associated with accessory abdominal organs known as "clasper siphons" which were believed for a long time to act merely as a reservoir of sea-water. In the course of an investigation on the function of these organs it was observed that the fluid obtained by flushing the siphon sacs with water gives a strong reaction with diazo reagents. Fluids obtained in this manner from five males were pooled, deproteinized, and the protein-free extract, representing a dry weight of 175 mg., subjected to further analyses. These have shown that the diazo-reactive substance is identical with serotonin (5-hydroxytryptamine). On the basis of optical activity measured at 275 mμ, the serotonin content of the protein-free extract was found to represent 6.2% of the dry weight. Determination based on absorption measurement of the purple-colored reaction product with 1-nitroso-2-naphthol gave a closely similar result, namely, 6.4%. Paper chromatograms sprayed with either diazobenzenesulfonic acid, para-dimethylaminobenzaldehyde, or ninhydrine, yielded spots with the same R_F value and color as pure serotonin.

Serotonin is known to possess the ability to stimulate uterine contractions. The participation of the fluid derived from the clasper siphons in the formation of fish seminal plasma, and the abundance of serotonin in that fluid suggest that, in the spiny dogfish at any rate, serotonin may play a role in the process of reproduction, either by influencing contractions of the female reproductive tract, thus facilitating sperm passage and fertilization, or by affecting the mechanism of copulation and ejaculation in the male.

Lipid-soluble sialic acid containing material in Arbacia eggs. L. WARREN AND R. HATHAWAY.

An Arbacia egg contains 3.8×10^{-4} μgm. bound sialic acid which can be completely released by hydrolysis for 2 hours at 80° C. in 0.1 N H_2SO_4. None of the sialic acid is normally free (*i.e.*, reactive in the thiobarbituric acid assay before hydrolysis) and there is none in the jelly coat.

At least two forms of sialic acid-containing substances are present in the egg, one of which is soluble in organic solvents. Approximately 25% of the total sialic acid can be readily extracted into n-butanol from a sea urchin egg homegenate. The lipid sialic acid complex is soluble in chloroform-methanol (2: 1) and in ether. The ether solubility suggests that the carboxyl group of the sialic acid residue may be blocked. Free sialic acids (N-acetylneuraminic acid and N-glycolylneuraminic acid) are insoluble in ether.

The sialic acid is released from the complex in one hour in 0.1 N H_2SO_4 at 80°. It is also released enzymatically by sialidase of *Vibrio cholerae*. The freed sialic acid has been purified by chromatography on a column of Dowex-1-acetate and has the following properties: It demonstrates the same color reactions as authentic material in the orcinol, Ehrlich, and thiobarbituric acid tests. It co-chromatographs on paper in two different solvent systems with N-glycolylneuraminic acid and separates completely from N-acetylneuraminic acid.

Other components of the lipid-soluble sialic acid-containing complex of Arbacia eggs described here are not known. The evidence obtained to date suggests that it differs from the gangliosides and other mucolipoproteins of animal brain.

Aided by RG-6234, National Institutes of Health, to Dr. Charles B. Metz.

Sialic acid in semen of Arbacia punctulata. L. Warren, R. Hathaway and J. G. Flaks.

Sialic acid is present in bound form in both the seminal plasma and sperm of Arbacia. Sialic acid appears to be present in at least two forms in seminal plasma: a readily hydrolyzable type (67%) which is cleaved within 30 minutes at 80° in 0.1 N H_2SO_4, and another form (33%) which takes four hours to hydrolyze under the same conditions. In one experiment 50% of seminal plasma sialic acid was enzymatically removed by the sialidase of *Vibrio cholerae*.

There are approximately 9.5×10^{-6} μgm. sialic acid in an Arbacia sperm. When sea water-washed sperm are treated with fertilizin, a soluble non-dialyzable sialic acid-containing material is removed from the sperm. The reaction appears to be complete within one minute and there is no further solubilization of sperm material unless more fertilizin is added. In one experiment four successive fertilizin treatments of a preparation of sperm led to the removal of 20% (12.0, 3.6, 3.2, 1.2%) of the sperm sialic acid. Heated fertilizin (80°, 5 minutes) was also active. Sialic acid substances in sperm may also be removed by detergents, such as sodium lauryl sulfate or cetyltrimethylammonium chloride. Four successive extractions of sperm by a 7.5×10^{-4} *M* sea water solution of sodium lauryl sulfate led to the removal of 82% (55.0, 18.8, 9.2, 5.9) of the sperm sialic acid, and to a loss of 25% of sperm dry weight. Approximately 12% of the material removed from the sperm could be accounted for as sialic acid. The sperm were still motile after the second extraction. Sperm also releases sialic acid-containing substances when suspended in distilled water and when incubated at 45° or higher, for 5 minutes.

Incubation of the solubilized sperm material at 80° for four hours in 0.1 N H_2SO_4 is required to cleave the sialic acid residue from the nondialyzable part. Only 5% of the sialic acid can be removed enzymatically. The sialic acid obtained by acid hydrolysis has been purified on a column of Dowex-1-acetate and gives the thiobarbituric acid, orcinol, and Ehrlich tests for sialic acid. On paper it co-chromatographed with N-glycolylneuraminic acid. The selective removal of materials from sperm by fertilizin appears to take place in a rapid non-enzymatic manner. The biological activities of the substances removed from sperm are described in an adjoining abstract.

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SULFUR METABOLISM IN THE LUGWORM, ARENICOLA CRISTATA STIMPSON[1], [2]

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Among the published reports on invertebrate biochemistry is a study by Robin (1954) using *Arenicola marina* L. Robin hypothesized that taurocyamine, a sulfur-containing guanidine derivative, was formed from taurine. Although the hypothesis has not been demonstrated experimentally, it constitutes a major part of the information presently available on sulfur metabolism in invertebrates.

Taurine in invertebrates generally has been regarded as a metabolically inactive compound. In *Arenicola*, however, since taurocyamine appears to be the phosphagen involved in energy transfers related to muscular activity, Robin's hypothesis requires an active metabolic role for taurine. Relatively high levels of taurine have been reported from a diversity of marine invertebrates. The fact that no definite role has been demonstrated for these high levels suggests that an organism such as *Arenicola*, which presumably both synthesizes and metabolizes taurine, might be a useful experimental tool for studying sulfur metabolism in invertebrates.

The present study was undertaken to determine the biochemical pathway leading to taurine formation in *Arenicola cristata* Stimpson, and to determine whether taurocyamine is synthesized by the route suggested by Robin or by some other mechanism.

MATERIALS AND METHODS

Animals

The specimens of *A. cristata* employed in this study were obtained from two sources. The Marine Biological Laboratory, Woods Hole, Massachusetts, supplied large specimens (50 to 200 gm. fresh weight). Worms of this size apparently are typical of the cold water environment in the Cape Cod area. Smaller individuals (5 to 20 gm. fresh weight) were obtained from the vicinity of St. Petersburg, Florida, through the courtesy of Dr. Victor G. Springer of the Florida State Board of Conservation Marine Laboratory.

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[2] The data presented here are extracted from the Ph.D. thesis of Walter Abbott.

[3] National Science Foundation Predoctoral Fellow in Biology, 1957–60.

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Compounds used

Taurocyamine was synthesized by reacting taurine with S-methylisothiourea in ammoniacal medium, according to the general method of Schütte (1943) as modified by Thoai and Robin (1954). Traces of taurine in the product were removed by chromatography on a column of Dowex 50 (H^+).

Hypotaurine (2-aminoethanesulfinic acid) was prepared by the method of Cavallini *et al.* (1955).

S^{35}-taurine was prepared from S^{35}-sodium sulfite (Abbott Laboratories) and β-bromoethylamine hydrobromide (Cortese, 1943). The product was purified by chromatography on a column of Dowex 50 (H^+).

S^{35}-methionine, S^{35}-cystine, and 3-C^{14}-serine were obtained from various commercial sources. S^{35}-cysteine was prepared by reduction of S^{35}-cystine (Lucas and Beveridge, 1940).

Extract preparation

Enzymatically active preparations were made by homogenizing living *A. cristata* with 10 volumes of 0.01 *M*, pH 7.3 phosphate buffer at temperatures of 0° to 4° C. Choice of pH was based on values given by Spector (1956) for *Arenicola* sp. The homogenates were centrifuged in the cold for 15 minutes at 1500 × *g* to remove large amounts of fibrous debris, then, after decantation, for 30 minutes at 25,000 × *g* to remove very small particulate components. The clear supernatant from the second centrifugation was quick-frozen immediately in Erlenmeyer flasks, and was lyophilized. A light, flesh-colored powder resulted. This powder was totally soluble in the phosphate buffer.

For determination of amino acids and related compounds, water-soluble fractions were prepared by homogenizing living worms in 80% ethanol and extracting by the method of Awapara (1948).

Before worms were used to make either type of extract, they were placed in enamel trays containing small amounts of clean sea water, in order to allow time for the worms to empty their digestive tracts. Attainment of this condition was assumed when fecal casts were not extruded for a period of at least 30 minutes.

Separation and identification of compounds

Dowex 50 (H^+), 200- to 400-mesh, a sulfonic acid-type cation exchanger, was used to separate polar compounds into two groups. Those compounds not retained on a column of the resin during elution with distilled water were termed "acidic compounds" in this study. Those compounds retained on the resin under these conditions were designated "basic compounds." Acidic compounds of interest here were taurine, taurocyamine, cysteinesulfinic acid, and cysteic acid. Basic compounds were displaced with 4 N NH_4OH.

For group separation, 3 gm. of Dowex 50 (H^+) were sufficient to treat an amount of water extract equivalent to at least 1 gm. fresh weight of tissue. In a small column were placed 1.5 gm. of resin. Another 1.5 gm. were slurried with the sample in a small beaker for 5 to 10 minutes. Contents of the beaker then were poured into the column. Beaker and column were rinsed liberally with distilled water.

Quantitative recovery of the acidic compounds was demonstrated by treating individual standards of these compounds in the manner just described. Recoveries in excess of 95% were obtained in all cases when 15 standard samples were treated as just outlined.

Thompson and Morris (1959) reported the chromatographic separation of mixtures of amino acids on a column of Dowex 50 (Na^+), using water or aqueous ethanol as solvent. This suggested separation of acidic compounds from *Arenicola* by chromatography on a column of Dowex 50 (H^+), using distilled water for elution. The column, 60 × 0.9 cm., was prepared from a slurry of reagent grade Dowex 50 (H^+) which had previously been washed to remove excess acidity. A column was used only once. The sample to be resolved was placed in the column in a volume of 1 ml. Elution was carried out at a rate of 5 to 6 ml. per hour. One-milliliter fractions were collected. Mixtures of standards could not be resolved until a soluble inorganic salt was added to the sample (25 to 50 mg./ml. NaCl), whereupon resolution became excellent (Fig. 1). The worm extracts contained sufficient inorganic material to obviate addition of salt. Schram and Crokaert (1957) obtained similar resolution of taurine and taurocyamine by eluting Dowex 50 (H^+) columns with 0.1 *M*, pH 2.5 citrate buffer.

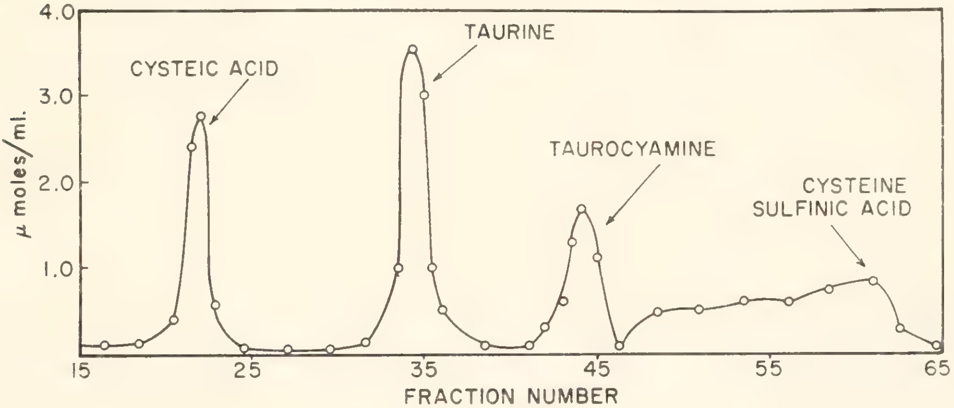

FIGURE 1. Elution of standards from 60 × 0.9 cm. Dowex 50 (H^+) column.

The four acidic components could be resolved by one-dimensional paper chromatography with phenol: water (72% v/v) as solvent. The basic components were separated by two-dimensional paper chromatography with phenol: water and 2,4-lutidine: water (65% v/v) as solvents. Papers used were Whatman Nos. 1, 4, or 3 MM.

Quantitative determinations of amino compounds were carried out by the method of Landua and Awapara (1949). Guanidylated entities were determined colorimetrically with diacetyl and α-naphthol (Rosenberg, Ennor and Morrison, 1956). Location of spots on papers was accomplished by two methods. Amino compounds were found by treatment of papers with 0.025% ninhydrin in acetone. Guanidino compounds were detected with the diacetyl reagent of Smith (1958), using the reagent as a spray. Table I shows the limits of detectability for various guanidine compounds when this reagent is used.

TABLE I

Detection limits for various guanidine compounds on paper chromatograms with the diacetyl spot reagent (Smith, 1958)

Compound	μg./cm.^{2} of paper*
Taurocyamine	0.75
Glycocyamine	1.06
Creatine	1.30
Arginine	2.00
Guanidine	1.59
Agmatine	2.00

* Values represent the smallest amount of a compound which was found to give a definite positive test on Whatman 3MM paper.

Hypotaurine was determined either by elution from Dowex 50 (H^+) columns with 2×10^{-3} N HCl (Bergeret and Chatagner, 1954), or by oxidation to taurine with hydrogen peroxide and determination as taurine. In the latter procedure, preliminary removal of endogenous taurine by Dowex 50 (H^+) was necessary.

Inorganic ions were determined as follows: (1) sulfites by the distillation-iodine titration method (Fromageot, Chatagner and Bergeret, 1948); (2) sulfates by the Versene technique (Welcher, 1958); and (3) chlorides by the Mohr technique.

Determination of endogenous levels of various substances was performed on a composite homogenate of 25 worms.

Experimental techniques

In vivo tracer studies were made by injecting worms intracoelomically with labeled metabolites. Leakage of compounds to the environment was found to be negligible during the time periods (12 to 36 hours) involved in these studies. Counting of aliquots of whole homogenates of injected worms showed recoveries of radioactivity which averaged 93.4% of the amount injected (Table II). Self-absorption was found to be negligible if the amount of material on the planchet was kept lower than 200 μg. per square centimeter. Hilchey, Cotty and Henry (1957) arrived at a value of 130 μg. per square centimeter, in similar studies.

The distribution of radioactivity into various compounds was determined in several ways—preparation of radioautographs from paper chromatograms, counting of material eluted from paper chromatograms, cocrystallization of metabolites

TABLE II

Recovery of radioactivity 24 hours after injection

Compound injected	Counts/minute		% recovery
	Injected	Found	
S^{35}-taurine	1.17×10^6	1.04×10^6	89.9
S^{35}-cysteine	8.00×10^4	7.50×10^4	94.0
S^{35}-methionine	2.20×10^7	2.19×10^7	99.5
3-C^{14}-serine	2.20×10^6	1.98×10^6	90.0

from extracts by addition of unlabeled carrier. A Nuclear-Chicago, thin window, gas flow counter was used for all counting.

In vitro studies were made with both fresh homogenates and with the lyophilized enzymatic preparation described previously. Enzyme preparations were dialyzed only when the reaction product of interest was present at a high endogenous level. All samples were prepared and adjusted to proper volume before addition of the enzyme preparation. Incubations usually were for one hour, and were performed at room temperature (22° to 23° C.).

TABLE III

Concentrations of free amino acids and related compounds in Arenicola cristata

Compound	Leucine equivalents/gm. of fresh weight*
Cysteine	3.92 ± 0.21
Homocysteine	0.52 ± 0.10
Aspartic acid	1.05 ± 0.05
Glutamic acid	1.73 ± 0.19
Serine	0.22 ± 0.05
Glycine	17.28 ± 0.09
Arginine	0.50 ± 0.07
Alanine	6.00 ± 0.22
Valine plus methionine	0.55 ± 0.03
Leucine	0.75 ± 0.03
Tyrosine	0.025 ± 0.020
Methionine sulfone	0.32 ± 0.08
Hypotaurine	0.48 ± 0.08**
Cysteinesulfinic acid	0.48 ± 0.05**
Taurine	3.28 ± 0.36**
Taurocyamine	5.06 ± 0.21**

* Values represent equivalent μmoles of leucine, except where otherwise indicated. All values represent mean values of four determinations, with standard errors, on aliquots of a homogenate of 25 worms.

** This value represents actual μmoles rather than leucine equivalents.

THE CHEMICAL COMPOSITION OF ARENICOLA

Vinogradov (1953) has summarized information on the chemical composition of *Arenicola marina* and *A. piscatorum*. These data are assumed to be applicable to *A. cristata*.

During the present study, a few additional determinations of inorganic components were made. Moisture content was determined on two worms. An average of 80.6% H_2O was found. Sulfate, determined on six worms, was 84.1 ± 5.3 μmoles per gram of fresh weight. Chloride was measured on three aliquots of the pooled extract of 25 worms. A level of 254 ± 1.9 μmoles per gram of fresh weight was established. Determinations on four aliquots of the same pooled extract by the colorimetric persulfate method showed total manganese to be less than 3.6×10^{-3} μmoles per gram of fresh weight.

Analyses for amino acids and other related materials were performed on the 25-worm extract. Results are given in Table III. Levels of basic compounds are reported as leucine equivalents. Hypotaurine and acidic compounds are reported directely in micromoles.

The status of cysteic acid as a metabolite is in question. It appears that in most cases where cysteic acid is found in extracts of normal tissues, it represents an artifact created by the extraction and isolation procedures. In the present work with *Arenicola,* it was found that cysteic acid always occurred when extracts were prepared at room temperature and were evaporated on the steam bath. When, however, extraction was carried out in the cold, and evaporation was done *in vacuo* without heat, no cysteic acid was detected. For this reason, cysteic acid is regarded as an artifact in *Arenicola cristata.*

TABLE IV

Qualitative studies in vivo using radioactive compounds

Compound found to be radioactive	Methods by which radioactivity was detected after injection of				
	S^{35}-taurine	S^{35}-methionine	S^{35}-cysteine	S^{35}-cystine	3-C^{14}-serine
Sulfate		1	1	1	
Homocysteine		2, 3			
Cystathionine		2	2	2	2, 4
Cysteine		2, 3	3	2	4
Cysteinesulfinic acid		2, 3, 4	3		
Cysteic acid		2, 3, 4	3		
Hypotaurine		5	5		
Taurine	3, 4	2, 3, 4		3	3

Methods:

1. Fifty mg. nonradioactive Na_2SO_4 added/ml. of sample. Sample acidified with HCl. Sulfate precipitated with slight excess of 10% $BaCl_2$ solution. Precipitate filtered, washed, and ignited for one hour at 1000° C. Ignited precipitate pulverized, distributed on planchet, and counted.

2. Compound extracted from sample by crystallization with nonradioactive carrier. Product recrystallized twice. Crystals dissolved. Solution evaporated on planchet and counted. Purity of final product checked by chromatography in four solvents.

3. Compound isolated by paper chromatography in several solvents. Spots eluted from papers. Eluates evaporated on planchets and counted.

4. Compounds isolated by paper chromatography. Radioautographs prepared from chromatograms.

5. Acidic compounds removed by Dowex 50 (H^+) treatment. Basic fraction oxidized with 30% H_2O_2. Oxidized material again separated with Dowex 50 (H^+). Taurine isolated from acidic fraction by paper chromatography in four solvents. Taurine spots eluted. Eluates evaporated on planchets and counted.

Robin's (1954) report of high arginase activity in homogenates of *A. marina* suggested a need for determination of the endogenous urea level in this worm. Manometric estimations with urease (Umbreit, Burris and Stauffer, 1957) showed, for the 25-worm extract, a urea level of 0.96 ± 0.013 μmoles per gram of fresh weight (four replications). Three standard urea samples were analyzed concurrently. The average value obtained for the standards was 101.6% (range: 97.2% to 105.3%) of the urea added.

Results and Discussion

Qualitative studies

These studies were conducted with the relatively large specimens of *A. cristata* obtained from Woods Hole Marine Biological Laboratory.

S^{35}-taurine was administered to a worm by intracoelomic injection. The worm was dissected after 24 hours, and all organs were shown to contain some radioactivity. Radioautographs prepared from chromatograms, and direct counting of material eluted from chromatograms, revealed no radioactivity in any compound other than taurine. This experiment indicated that taurocyamine, although known to contain sulfur, apparently was not formed from taurine. Therefore, a tracer study of sulfur metabolism in *A. cristata* was undertaken.

Radioactive compounds were given by injection and after 12 to 36 hours the animals were homogenized and extracts made. The results of the tracer studies and the methods used to obtain these results are given in Table IV. The labeled intermediates found did not suggest any qualitative departure from the basic sulfur catabolism scheme known for vertebrates. Taurine was formed from methionine or cystine, presumably via decarboxylation of cysteinesulfinic acid to hypotaurine with subsequent oxidation of the latter. Cysteine and cystine were metabolically interconvertible, although the rate and nature of this conversion are not known. A cystathionine pathway from methionine to cysteine exists and, as is also known in the vertebrate, involves serine. In no case was any radioactivity detected in taurocyamine, even 36 hours after injection of cystine.

Quantitative studies

These studies were conducted with the small specimens of *A. cristata* obtained from the St. Petersburg, Florida, area. In all studies using labeled compounds, total recovery of radioactivity was assumed. With this assumption, data regarding distribution of radioactivity may be regarded as representing the distribution of the entire metabolic pool of the injected compound. Because of the complete overlap between elution peaks from Dowex 50 (H^+) columns for sulfate and for cysteic acid, radioactivity levels for these compounds are pooled in all values given for distribution of radioactivity after injection of S^{35}-labeled compounds.

Tracer studies showed that *A. cristata* converted approximately 50% of injected S^{35}-cysteine into oxidized, strongly acidic entities within 12 hours. With cystine, the process was much slower, only about 18% being oxidized in 12 hours. The 50% level was attained with cystine by 36 hours.

When 3-C^{14}-serine was injected into the worms, almost 60% of the total radioactivity appeared within 24 hours in strongly acidic compounds, *i.e.*, those which passed through the Dowex 50 (H^+) column. Most of these components showed no reaction with ninhydrin. An estimated 12% of the total radioactivity was associated with strongly acidic sulfur-containing compounds (Table V). This was about the same amount of incorporation as was obtained in 12 hours with S^{35}-cysteine, excluding inorganic sulfate.

With methionine, only 17.8% of the total radioactivity appeared in the acidic fraction in 24 hours. Of this, 13.5% was in sulfate plus cysteic acid, 2.7% in taurine plus an unidentified compound, and 1.6% in cysteinesulfinic acid. Presumably, the rate of demethylation of methionine to form homocysteine was the limiting factor in this system.

Relatively high percentages of radioactivity were incorporated into sulfate, as compared with other sulfur compounds, after injection of S^{35}-cysteine or S^{35}-cystine (Table V, Fig. 2). These percentages are of the same order as those reported from other sources (Kay and Entenman, 1959; Skarzynski, Szczepkowski and Weber, 1959; Eldjarn, Pihl and Svedrup, 1956; Tarver and Schmidt, 1942).

TABLE V

Distribution of radioactivity among acidic compounds in Arenicola cristata following injections of labeled compounds

Injected compound	Fraction	Counts/minute/gm. of fresh weight	Distribution of total radioactivity %
S^{35}-cysteine (extraction 12 hours after injection)	Whole extract	21,360	100
	Acidic portion	11,070	51.83
	Sulfate + cysteic acid	8,460	39.61
	Taurine	490	2.29
	Cysteinesulfinic acid	1,208	5.66
	Compounds X_1 and X_2	912	4.27
S^{35}-cystine (extraction 12 hours after injection)	Whole extract	23,925	100
	Acidic portion	4,250	17.77
	Sulfate + cysteic acid	3,938	16.46
	Taurine	155	0.65
	Cysteinesulfinic acid	79	0.33
	Compounds X_1 and X_2	78	0.33
S^{35}-cystine (extraction 36 hours after injection)	Whole extract	21,510	100
	Acidic portion	10,823	50.32
	Sulfate + cysteic acid	8,708	40.48
	Taurine	1,462	6.80
	Remainder	653	3.04
S^{35}-methionine (extraction 24 hours after injection)	Whole extract	438,000	100
	Acidic portion	78,000	17.8
	Sulfate + cysteic acid	59,300	13.5
	Taurine + X_1	11,500	2.7
	Cysteinesulfinic acid	7,200	1.6
3-C^{14}-serine (extraction 24 hours after injection)	Whole extract	14,710	100
	Acidic portion	8,600	58.5
	Taurine*	1,860	12.6
	Compounds not containing sulfur	6,740	45.9

* Since many highly acidic carbon compounds emerge at the front during elution of the Dowex 50 (H^+) columns, radioactivity associated with cysteic acid cannot be isolated. Cysteinesulfinic acid shows no radioactivity. Therefore, it is assumed that cysteic acid also is not radioactive and that all radioactivity appearing in sulfur-containing strongly acidic compounds is associated with taurine.

Fromageot (1953), Kearney and Singer (1953a, 1953b), Chatagner *et al.* (1952) and others have all emphasized that the most important pathway of sulfur from cysteine to sulfate is through transamination between cysteinesulfinic acid and α-ketoglutaric acid to form glutamic acid and an unstable intermediate, β-sulfinylpyruvic acid. The latter decomposes into sulfite, which is oxidized to sulfate, and into pyruvic acid, which by transamination with glutamic acid regenerates α-ketoglutaric acid and forms alanine.

It has been demonstrated that both enzymatic and non-enzymatic oxidation of sulfite to sulfate occur in mammals (Heimberg, Fridovich and Handler, 1953; Fridovich and Handler, 1956). The enzymatic process is heat-labile. When sodium sulfite solutions were incubated with the soluble enzyme preparations from *A. cristata,* the results in terms of sulfite remaining after one hour, although somewhat erratic, indicated that no decrease in activity was effected by boiling the enzyme preparation. Thus, the enzymatic mechanism may be of little importance in *Arenicola,* or may be absent.

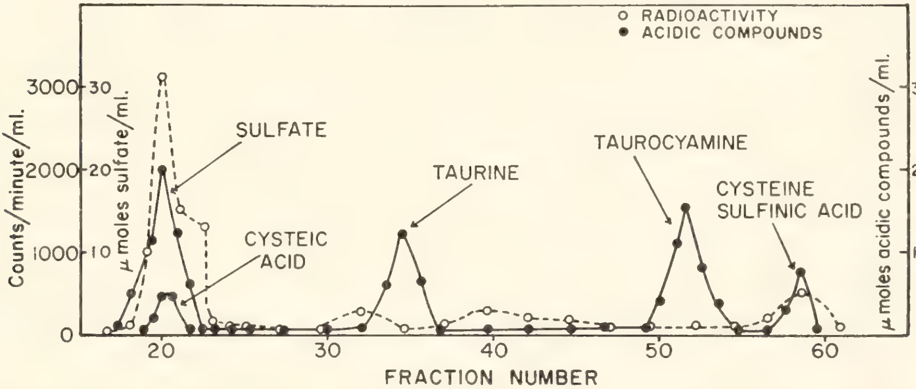

FIGURE 2. Acidic components from 1 gm. of *Arenicola cristata* 12 hours after injection of S^{35}-cysteine. Fractions are 1-ml. portions of eluate from 60 × 0.9 cm. Dowex 50 (H^+) column.

The activity of the cysteinesulfinic acid-α-ketoglutaric acid transaminase system *in vitro* may be evaluated from Table VI, which presents the most consistent series of experimental results obtained in several trials of this system. The values reported for glutamic acid represent increases above the endogenous level for this substance. There seems little reason to doubt the formation of sulfate via the transaminase-β-sulfinylpyruvate pathway.

The formation of hypotaurine in the mammal by decarboxylation of cysteinesulfinic acid has been established for some time (Bergeret and Chatagner, 1952; Bergeret, Chatagner and Fromageot, 1952; Awapara and Wingo, 1953). Pyridoxal phosphate has been shown to act as coenzyme for this reaction and to resist dialysis, indicating that it is tightly bound to the apoenzyme (Bergeret and Chatagner, 1952; Blaschko and Hope, 1954; Hope, 1955). In the present study, hypotaurine was found to occur in *A. cristata* at an endogenous level of 0.5 μmole per gram of fresh weight. Attempts to show decarboxylation of cysteine-sulfinic acid *in vitro* gave highly variable results. Whole homogenates and solutions of the lyophilized soluble fraction of the worm were tested. Pyridoxal phosphate was added to samples at levels of 4 to 8 μg. per milliliter. In all manometric experiments carbon dioxide was released. Chromatography of reaction mixtures after 60-minute incubation periods revealed distinctly more hypotaurine in reactive samples than in controls, based on comparison of colors and sizes of spots on ninhydrin-developed papers. Colorimetric estimation showed that an erratic increase in hypotaurine occurred in samples as compared with controls. The reaction was heat-labile.

TABLE VI

*Cysteinesulfinic acid-α-ketoglutamic acid transamination**

Sample number	ml. of extract added	μmoles of cysteinesulfinic acid added	μmoles of α-ketoglutaric acid added	μmoles of glutamic acid formed/gm. of fresh weight/hour
1	1.0**	25	25	0.0
2	1.0**	25	25	0.0
3	0.0	25	25	0.0
4	0.0	25	25	0.0
5	1.0	25	0	0.0
6	1.0	25	0	0.0
7	1.0	0	25	0.0
8	1.0	0	25	0.0
9	1.0	25	25	12.1
10	1.0	25	25	6.0
11	1.0	25	25	7.2
12	1.0	50	25	10.5
13	1.0	50	25	9.9
14	1.0	50	25	10.5
15	1.0	75	25	11.0
16	1.0	75	25	11.0
17	1.0	75	25	11.6
18	1.0	100	25	11.0
19	1.0	100	25	10.5
20	1.0	25	50	8.3
21	1.0	25	50	8.3
22	1.0	25	75	9.9
23	1.0	25	75	9.3
24	1.0	25	100	9.3
25	1.0	25	100	9.9
26	1.0	50	50	12.7
27	1.0	50	50	12.7
28	1.0	100	100	15.0
29	1.0	100	100	17.7
30	2.0	25	25	15.0
31	2.0	25	25	12.2
32	2.0	25	25	12.7
33	3.0	25	25	19.4
34	3.0	25	25	17.2
35	3.0	25	25	17.2

* Conditions: All solutions prepared in 0.01 *M* phosphate buffer, pH 7.3. Total volume: 5.0 ml. Each ml. of extract contained 50 mg. of lyophilized soluble fraction of *A. cristata*. Aerobic incubation of samples for 120 minutes at temperatures of 22° to 23° C. Acids neutralized with NaOH prior to making stock solutions.

** Extract boiled for 5 minutes before addition to sample.

TABLE VII

*Oxidation of hypotaurine to taurine**

Sample number	ml. of homogenate added	Molarity of hypotaurine	μmoles of taurine formed/hour/gm. of fresh weight
1	1.0	0.0040	12.0
2	1.0	0.0040	12.5
3	1.0	0.0040	13.0
4	1.0	0.0035	11.5
5	1.0	0.0035	11.5
6	1.0	0.0035	12.0
7	1.0	0.0030	10.0
8	1.0	0.0030	11.5
9	1.0	0.0030	11.0
10	1.0	0.0025	8.5
11	1.0	0.0025	10.5
12	1.0	0.0025	9.0
13	1.0**	0.0025	8.0
14	1.0**	0.0025	8.5
15	2.0	0.0025	9.0
16	3.0	0.0025	9.0
17	0.0	0.0025	0.0

* Conditions: All solutions prepared in 0.01 *M* phosphate buffer, pH 7.3. Total volume: 11.0 ml. Fresh homogenate of *A. cristata*: 1 ml. equivalent to 200 mg. of fresh weight. Aerobic incubation of samples for 60 minutes at temperatures of 22° to 23° C. Hypotaurine neutralized with NaOH before stock solution was prepared. Taurine determined by separation on Dowex 50 (H^+), columns followed by ninhydrin treatment of aliquots of filtrates.

** Homogenates boiled for 5 minutes before addition to sample.

The oxidation of hypotaurine to taurine was studied with fresh whole homogenates and with the soluble enzyme extract. The reaction appeared to be non-enzymatic; it was not hindered by prolonged boiling (up to 20 minutes) of homogenates and extracts. Table VII shows the most consistent results obtained from the series of experiments on this reaction. Values for taurine formation are based on increases above the endogenous level. A reciprocal plot of this experiment was derived by least squares analysis. Statistical testing indicates that the line does not pass through the origin ($0.02 > p > 0.01$). Other less extensive experiments gave regression lines which were not significantly different from lines passing through the origin. It is postulated that the reaction is non-enzymatic because of its total insensitivity to prolonged heating. Metal ion catalysis may be involved here since the presence of the worm homogenate or extract is necessary to the reaction. It is further suggested that the reciprocal plot should not pass through the origin, as there is undoubtedly some substrate level at which catalyst saturation occurs and the reaction becomes zero order.

Repeated attempts were made to demonstrate transamidination between arginine and a variety of acceptor compounds—taurine, hypotaurine, cysteine-sulfinic acid, cysteic acid, cysteine, cystathionine, and mercaptoethylamine. All results were negative. Care was taken to add sufficient arginine (20 or more μmoles per milliliter of sample) so that arginase activity could not seriously deplete the supply of the amidine group donor during incubation.

The experiment with the S^{35}-taurine injection was repeated with taurine of higher specific activity. Twelve hours after injection, the animal was homogenized and the homogenate was fractionated by chromatography on the Dowex 50 (H^+) column. When the fractions were checked for radioactivity, a slight amount of radioactive sulfur was detected in the taurocyamine. On the assumption of no leakage of compounds to the environment, and with the endogenous concentration of taurine known, it was calculated that only 0.60 μmole of taurocyamine—0.10 μmole per gram of fresh weight—had been formed in 12 hours. The correlation of taurocyamine content with radioactivity in 1-ml. fractions eluted from the column is shown in Figure 3.

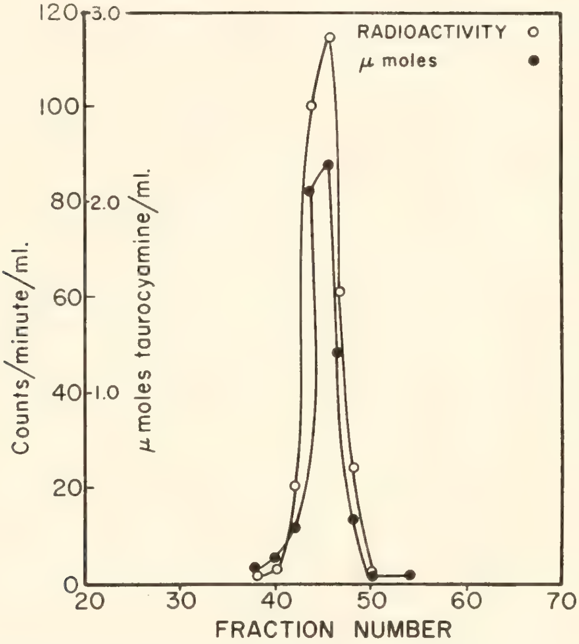

FIGURE 3. Taurocyamine fractions from 1 gm. of *Arenicola cristata* 12 hours after injection with S^{35}-taurine. Fractions are 1-ml. portions of eluate from 60 × 0.9 cm. Dowex 50 (H^+) column.

As soon as this information was obtained, a solution of moderately radioactive S^{35}-taurine was prepared for use in *in vitro* studies. Three experiments—two with fresh homogenates of *A. cristata* and one with a lyophilized soluble fraction—were performed in an attempt to obtain transamidination between arginine and taurine. Success was achieved with only one sample, which was part of the experiment using the lyophilized preparation. After incubation for 60 minutes, 0.004 μmole of taurocyamine was formed by this sample, which contained 100 μmoles of arginine, 5 μmoles of taurine (representing 2335 counts per minute), and an amount of extract equivalent to 0.61 gm. of fresh weight of worm tissue. Total volume was 5.0 ml.

From the *in vivo* results, it might be assumed that the slow rate of taurocyamine formation resulted from competition between arginase (Robin, 1954) and transamidinase for the limited arginine supply. The over-all endogenous level of arginine was about 0.5 μmole per gram of fresh weight, all of which seemed to occur in the wall of the digestive tract. However, the assumption of competition was not supported by *in vitro* results, as sufficient arginine was present to supply both systems adequately. Dr. James B. Walker (personal communication) has speculated that his transamidinase preparations from mammalian tissues and from *Streptomyces griseus* probably would show similar low levels of activity toward almost any suitable amidine group acceptor.

Summary and Conclusions

The pattern of sulfur metabolism in *Arenicola cristata* was examined. Qualitatively, it differs little from the pathway known in mammals, except in one of the terminal reactions. This reaction leads to incorporation of cysteine sulfur and, hence, of methionine sulfur into the compound taurocyamine by transamidination between taurine and arginine, as suggested by Robin (1954).

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HISTOLOGICAL STUDIES ON THE DIGESTIVE SYSTEM OF A STARFISH, HENRICIA, WITH NOTES ON TIEDEMANN'S POUCHES IN STARFISHES[1]

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In starfishes of the genus *Henricia* (Family Echinasteridae), the digestive tract is a complex system presenting many highly specialized features and contrasting markedly with the much simpler digestive organs of forms such as *Asterias* or *Pisaster*. In consequence of a number of descriptive and experimental studies, structural and functional aspects of feeding and digestion in Asteriidae are reasonably well understood; in contrast, very little is known of the details of structure and function in the alimentary tract of *Henricia*. From the functional standpoint, for instance, we remain in ignorance of the normal food of *Henricia* (Mortensen, 1927, p. 120), and of its mode of feeding. Anatomy and histology have received somewhat more attention: Cuénot (1887) devotes some descriptive remarks to the digestive system in *Cribella oculata* (= *Henricia sanguinolenta*), and Hayashi (1935) includes sections on the structure and histology of the digestive tract in his article on the anatomy of a Japanese variety of *H. sanguinolenta* which later (1940) he considers a separate species, *H. ohshimai*. Hayashi's account is reasonably accurate as far as it goes, but in terms of the digestive tract it is disappointingly superficial, omitting to mention several conspicuous features and relationships that are both interesting and significant. The present study was undertaken originally as a necessary foundation for contemplated experimental studies on the digestive tract. It presents a more nearly complete description of anatomical and histological details, seeks to combine these with histochemical characteristics of parts of the digestive system as possible clues to function, and draws attention to unique features of the system unaccountably omitted from previous descriptions.

Prominent among these are the structures associated with the pyloric caeca which Ludwig and Hamann (1899) named "Tiedemann's pouches" in recognition of their discoverer. In his epochal monograph of 1816, which laid the foundations of our modern knowledge of echinoderm morphology, Friedrich Tiedemann briefly described a detail of structure in *Astropecten auranciacus,* as follows (p. 48): "On the lower surface of each caecum there is a small, elongate, and hollow appendage, which commonly contains a yellowish fluid. Perhaps this appendage is a kind of secretory organ." Cuénot (1887) called attention to the singular development of these structures in starfishes of the families Asterinidae and Echinasteridae (p. 34): ". . . the median sac is considerably elongated and forms a voluminous reservoir, which, in the natural position of the animal, is situated below the radial caecum; this reservoir runs about half or three-quarters the

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length of the caecum and is marked by regularly spaced oblique folds; it opens widely into the gastric sac, of which it is nothing more than a continuation." (See also Cuénot's Plate II, Figures 11 and 18.)

Considering the conspicuous and distinctive nature of these structures in the starfishes that possess them, it is surprising that Tiedemann's pouches (or Tiedemann's diverticula, as they are also called) have attracted so little attention with regard to either structural details or functional possibilities. Vogt and Yung (1888), referring to the relatively small pouches in *Astropecten,* mention that solid food has been observed in the pouches but not in the pyloric caeca themselves. Irving (1924), in his discussion of ciliary currents in *Patiria miniata,* describes Tiedemann's pouches as the median ducts of the caeca which bear the glandular pockets on their aboral walls. Misled by this description, I later (Anderson, 1953) used the same terminology in discussing the pyloric caeca of *Asterias forbesi.* It must be noted that the caeca of the family Asteriidae do not possess Tiedemann's pouches and that where they do occur, these pouches are not the median ducts themselves but form extensive appendages on the oral sides of the median ducts. Finally, Hayashi (1935) pays practically no attention to these conspicuous structures in his anatomical study of *Henricia,* saying only (p. 9), "Each caecum has a spacious median canal which is elongated dorsoventrally in cross-section, and the wall is thin and folded."

We thus appear to lack altogether any detailed information on structure and function in Tiedemann's pouches. Accordingly, in the present study some emphasis is placed upon the anatomical and histological features of Tiedemann's pouches in *Henricia,* and upon the very considerable differences that exist between the pouches in *Henricia* and those in *Patiria,* a member of the other family (Asterinidae) in which such pouches typically occur, according to Cuénot (1887). Where pertinent, brief remarks are included concerning preliminary studies of the pouches in *Asterina gibbosa, Astropecten irregularis* and *armata,* and *Linckia guildingi.*

These studies were begun during tenure of a John Simon Guggenheim Memorial Fellowship in 1958–59, at the Hopkins Marine Station of Stanford University, Pacific Grove, Calif., and continued at the Marine Biological Laboratory, Woods Hole, Mass. The generous support of the Guggenheim Memorial Foundation, and the hospitality and cooperation of the Director and Staff of the Hopkins Marine Station, are hereby gratefully acknowledged. I also acknowledge with thanks the aid of the following, who provided specimens of starfishes from the indicated areas: Dr. F. S. Russell, Plymouth; Dr. R. A. Boolootian, Los Angeles; Aage Möller Christensen, Helsingör; and Jonathan Green, Hawaii.

MATERIALS AND METHODS

Small to moderate-sized specimens of *Henricia leviuscula* were collected beneath stones in the intertidal zone at Point Piños, Pacific Grove, Calif. They were maintained in running sea-water in one-gallon jars provided with escape-proof collars of plastic screening. The specimens could not be induced to feed upon any of the variety of items readily accepted by *Patiria* and other species, but as they remained in apparently vigorous condition for periods of two months or more, the possibility exists that they were feeding unobserved upon suspended particulate material in the water, or upon the algal and bacterial film on the walls of their containers. Comparative and confirmatory observations were made at Woods Hole on locally-collected specimens of *Henricia sanguinolenta* maintained under similar conditions. It is apparent that the two species do not differ significantly in the details of their internal anatomy, and the results to be reported are composite.

Gross anatomical studies involved specimens dissected after treatment with $MgCl_2$ (8% in tap water) to prevent movement or autotomy. For histological examination, tissues were fixed in Helly's fluid, washed, dehydrated, imbedded in paraffin, and sectioned serially at 7 to 10 μ; or fixed in Baker's formol-saline, post-chromed 24 hours in potassium bichromate, imbedded in gelatin, and sectioned on the freezing microtome. Tissues that included parts of the body wall were decalcified by soaking for approximately a week in 5% aqueous disodium ethylenedinitrilo-tetraacetate (EDTA), a chelating agent, between fixation and dehydration steps.

For general study and orientation, paraffin sections were stained with Harris' hematoxylin and eosin. For the demonstration of muscle fibers, connective tissue, cell membranes, flagella and their basal bodies, and secretion granules, excellent results were obtained by the use of Mallory's phosphotungstic acid hematoxylin. Glycogen and other polysaccharide compounds were demonstrated by a periodic-acid-Schiff technic, controlled by salivary digestion, and metachromatic substances were stained by overnight exposure to very dilute solutions of toluidine blue. Steedman's Alcian Blue technic, as given by Pearse (1953), was employed for staining acid mucopolysaccharides. The frozen sections of gelatin-imbedded material were colored with Sudan black, counterstained with carmalum, to reveal lipid deposits.

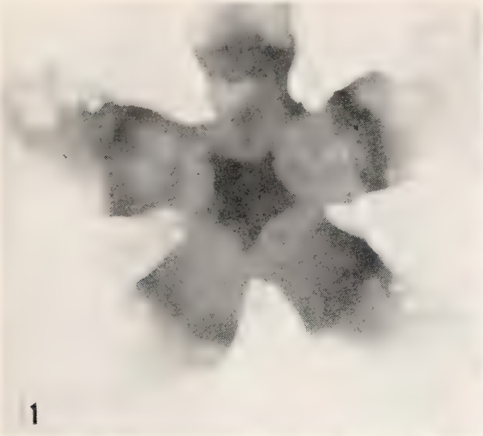
Observations

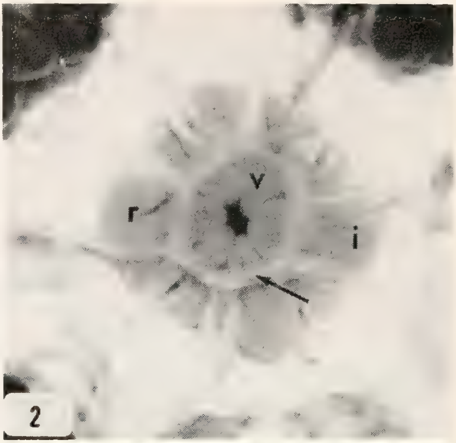
A. Anatomy

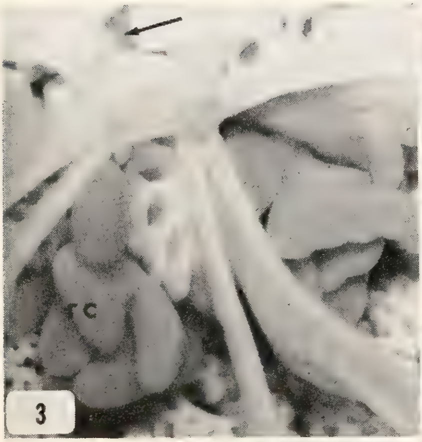
As in starfishes generally, the digestive tract of *Henricia* is essentially a tube, running from mouth to anus in the short vertical axis of the body, divided into specialized successive regions termed cardiac stomach, pyloric stomach, and intestine (esophagus and rectum are additionally distinguished by many authors). The pyloric stomach gives off a pair of glandular appendages, the pyloric caeca, into each ray, and the intestine (or rectum) bears an exceptionally well-developed group of sacculate appendages, of unknown function, termed rectal caeca or intestinal caeca.

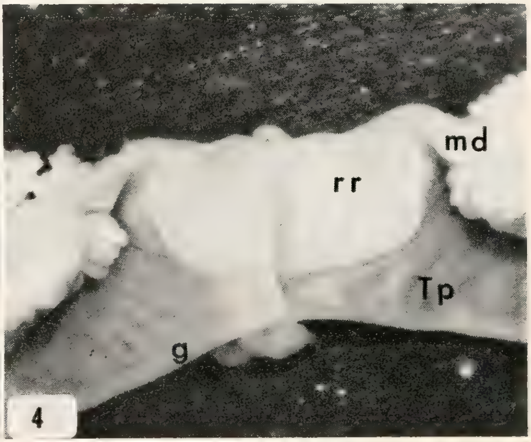
Viewed from below, as in Figure 1, the mouth appears as a stellate opening bounded by 5 peristomial lobes which approach each other centrally. When the animal is undisturbed, the mouth is usually partially open, as in this photograph. Hayashi (1935) terms the cardiac stomach, into which the mouth opens, "rudimental." It is relatively small, compared with that of *Asterias*, for example, and lies wholly within the circular frame bounded by the proximal ambulacral ossicles (Fig. 2). The stomach consists largely of a series of structures termed "esophageal pouches" by Cuénot (1887). There are 10 of these, 5 radial and 5 interradial in position; each radial pouch lies just medial to the large proximal ambulacral ossicle in its ray and is movably bound to this ossicle by a pair of

Figures 1 through 27 illustrate features of the digestive system in *Henricia;* Figures 28 through 31 show details of Tiedemann's pouches in *Patiria miniata.*









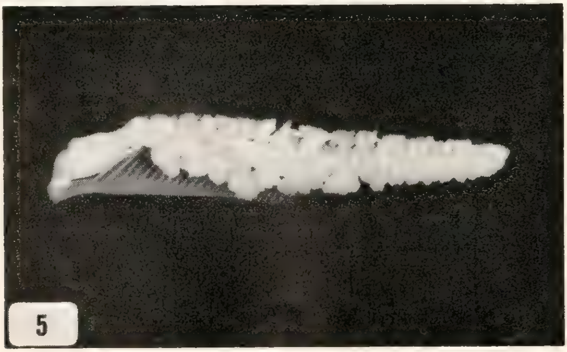


FIGURE 1. Oral view of living specimen, showing partially open mouth surrounded by peristomial membrane. Approximately 20 ×.

FIGURE 2. Cardiac stomach from above, after removal of all aboral parts by transecting the pyloric stomach at the line indicated by the arrow. Note radial pouches (*r*) ; interradial

stout, fibrous retractor strands. From the same origins a pair of longer strands pass upward to insertions higher on the wall of the stomach. Hayashi remarks on the thick and compact appearance of these retractor strands in contrast to the highly branched fibers in *Asterias;* in *Henricia,* although short strands do extend to the outer surface of the adjacent esophageal pouches, these are neither so numerous nor so extensive as those forming what has been termed the intrinsic retractor mechanism in *Asterias* and *Patiria* (Anderson, 1954, 1959).

Above the interradial pouches the wall of the stomach folds inward longitudinally to form 5 or 6 (usually 5) large, centrally-directed bulges that almost occlude the lumen of the stomach but leave a narrow, stellate central passageway (Fig. 2). These swollen vesicles are separated from one another by deep, radiating folds which are restricted lateral extensions from the angles of the central passage and which, in consequence of their radial positions, form conspicuous gutters leading upward from the cavities of the radial esophageal pouches. Above the level of these alternating vesicles and gutters the stomach is encircled by a slender fibrous girdle, into which the longer retractor strands from each ray insert. There is no real constriction marking the separation between cardiac and pyloric portions of the stomach, and in the absence of other landmarks the regions above this circumferential girdle will be considered as belonging to the pyloric stomach. This is in agreement with the situation in *Patiria,* where a similar fibrous girdle has been taken to mark the upper limit of the cardiac stomach (Anderson, 1959).

Just above the level of the girdle, the pyloric stomach tapers somewhat, and its walls are perforated by a marginal opening at each of its radial angles. Each of these 5 openings leads into a duct which immediately bifurcates, giving rise to pale, translucent, cylindrical branches forming the tubular proximal channels that expand vertically into the two Tiedemann's pouches in its ray (Figs. 3, 4, 5). It should be noted that each marginal passage constitutes, in effect, a continuation of one of the radial gutters leading upward between the vesicles of the cardiac stomach, from a radial esophageal pouch.

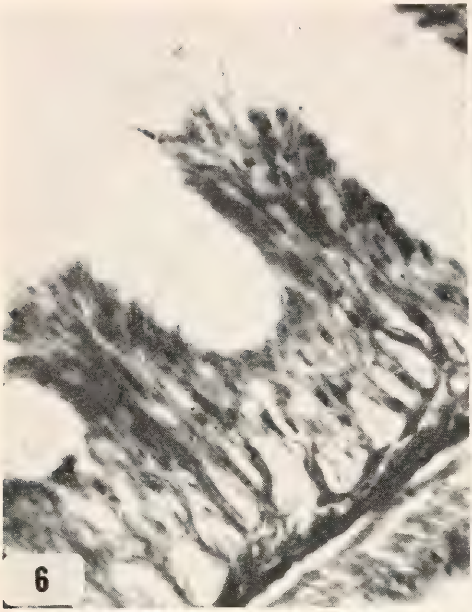
Above the marginal openings, the pyloric stomach forms 10 radiating branches, rather deep in the vertical dimension, two leading towards each ray. In each of these branches the side walls evaginate and are thrown into coarse folds, forming what I shall call radial reservoirs, with creamy-white, opaque walls. The radial reservoir tapers upward and outward, narrowing as the underlying Tiedemann's pouch expands, and becomes the median duct of one of the paired pyloric caeca

pouches (*i*); vesicles (*v*) surrounding the central aperture; and the stout retractor harness sending strands over the radial pouches to attach on the circumferential girdle (*arrow*) marking the boundary between cardiac and pyloric stomach. Approximately 15 ×.

FIGURE 3. Aboral parts of the digestive system viewed from below. Arrow indicates the central lumen of the pyloric stomach, from which the caecum-pouch complexes radiate in pairs (for details of these parts, see following figures). Note the well-developed rectal caeca (*rc*). Approximately 15 ×.

FIGURE 4. Basal parts of one pair of caecum-pouch complexes, cut off from central attachments and spread apart to show components. Tiedemann's duct widens into Tiedemann's pouch (*Tp*), with gutter (*g*) forming its oral margin; above, the median duct (*md*) of the caecum widens into the thick-walled radial reservoir (*rr*) by which it connects to the pyloric stomach. Approximately 15 ×.

FIGURE 5. A single caecum-pouch complex, cut off at left from its attachment to the pyloric stomach, showing relationship between Tiedemann's pouch (note parallel channels traversing its walls), radial reservoir, median duct, and lateral diverticula. Approximately 5 ×.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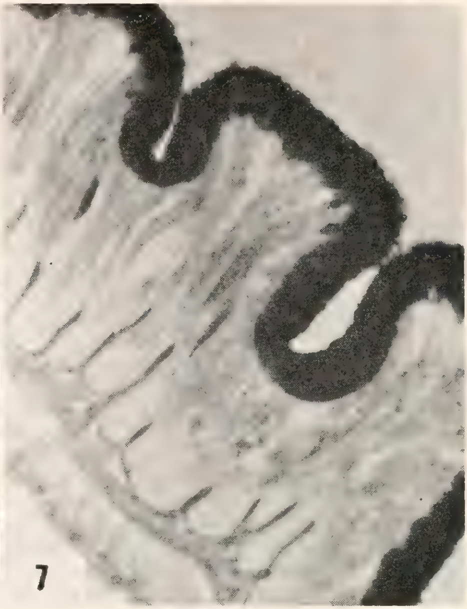
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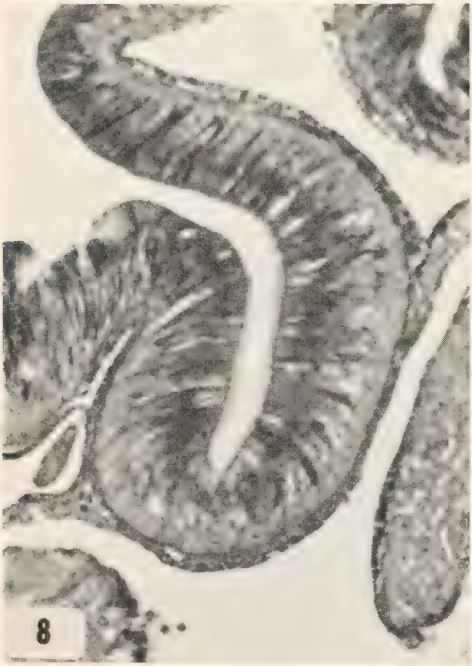
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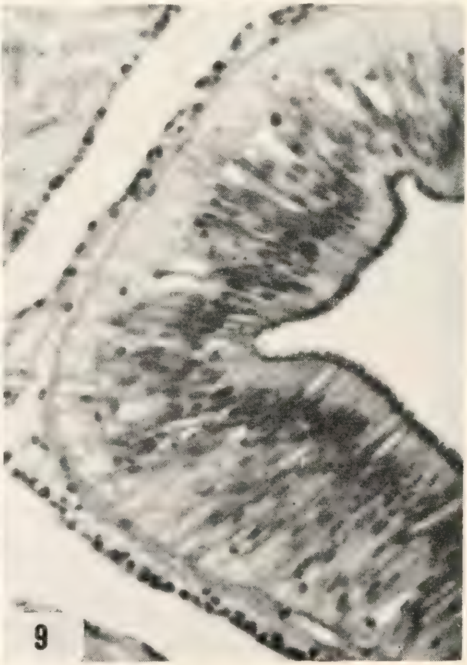
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FIGURE 6. Extreme lower end of cardiac stomach ("esophagus"); note the heavy partitions extending upward from the connective-tissue layer; the tall epithelial cells with apparently empty distal ends; and the strands of secretory granules marking locations of the few zymogen cells in this area. Phosphotungstic acid hematoxylin; 470 ×.

(Figs. 4, 5). The first of the lateral diverticula of the pyloric caecum leaves the median duct somewhat beyond the end of the radial reservoir.

The figures clearly demonstrate that the pyloric caecum-Tiedemann pouch complex is dual in nature. The aboral part, consisting of the median duct with its many lateral diverticula, has its affinities with the radial reservoir, of which it appears to be a continuation. Tiedemann's pouch, on the other hand, altogether different in gross appearance, originates almost at the junction between cardiac and pyloric portions of the stomach and is evidently closely related to the lower region. The two ducts, the median duct and what we may call Tiedemann's duct, accompany each other and are closely bound together, but their cavities are completely separated by a continuous partition. Passing outward, the lower duct becomes a gutter that forms the oral margin of the deep, narrow pouch, and the body of the pouch itself extends between this gutter and the median duct of the pyloric caecum above. Throughout its length, the side walls of the pouch are traversed by a series of diagonal, parallel lines, giving the external appearance of the "regularly spaced oblique folds" remarked by Cuénot (1887). Proceeding outward, the depth of the pouch decreases gradually, and at its outer end (about two-thirds the length of the caecum from its base) the oral gutter enters the floor of the median duct of the caecum (Fig. 5).

The roof of the pyloric stomach shows folds corresponding to the radial branches of the walls; these converge centrally upon the opening of the intestine. I can add nothing to Hayashi's description of the anatomy of this portion of the digestive tract, or of the rectal caeca which are extraordinarily well developed in *Henricia* (Fig. 3).

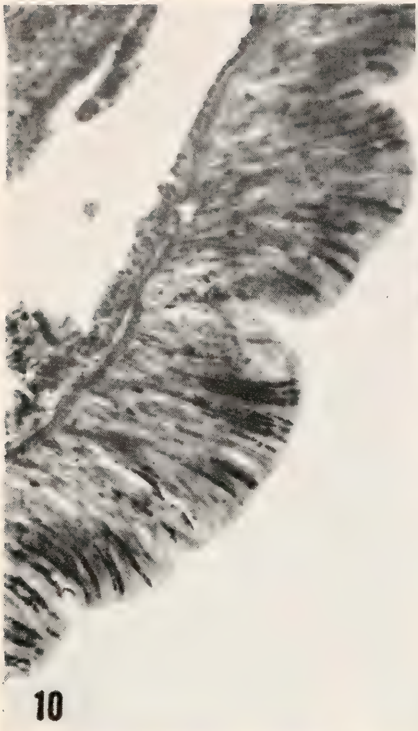
B. Histology

Histologically, the gut wall of *Henricia* presents the general features that appear to be standard throughout the Asteroidea. The usual tissue layers are present—peritoneum, muscular layers, connective-tissue layer, nerve plexus layer, and lining epithelium—and occupy the same relative positions as in other starfishes. The chief inadequacies of Hayashi's otherwise good account describing these layers involve his omission of significant details concerning the lining epithelium. This is basically a very tall layer, composed of what may be termed "typical" cells—long, slender cells, crowded together, with their ovoid nuclei lying at varying levels in the epithelium; each cell is provided, usually, with a single flagellum, springing from an apical basal body that sends a tapering fibril downward towards the nucleus. Better developed in some parts of the stomach than in others, longitudinal supporting fibrils also run through the basal portions of the

FIGURE 7. Adjacent section, showing intense metachromatic staining with toluidine blue in the distal areas that do not stain with PTAH. The faint coarse granules scattered in the epithelial cells are also metachromatic, as are the contents of the mucous goblets. 470 ×.

FIGURE 8. Section across a radial pouch, low in the cardiac stomach, showing tall, flagellated epithelium and relative scarcity of secretory cells in the pouch itself. Note also the relationship of the retractor strands to the connective-tissue layer in this region. Periodic-acid-Schiff, Weigert hematoxylin, fast green; 235 ×.

FIGURE 9. Detail of epithelium in a radial pouch. Note the tall, crowded, flagellated cells with their conspicuous brush border, and the well-developed nerve plexus layer through which bases of the epithelial cells extend to attach on the connective-tissue layer. Alcian Blue-carmalum; 470 ×.



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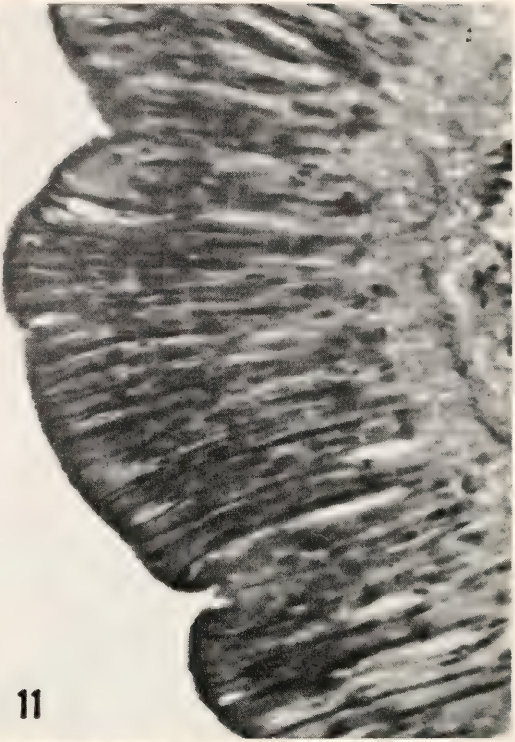

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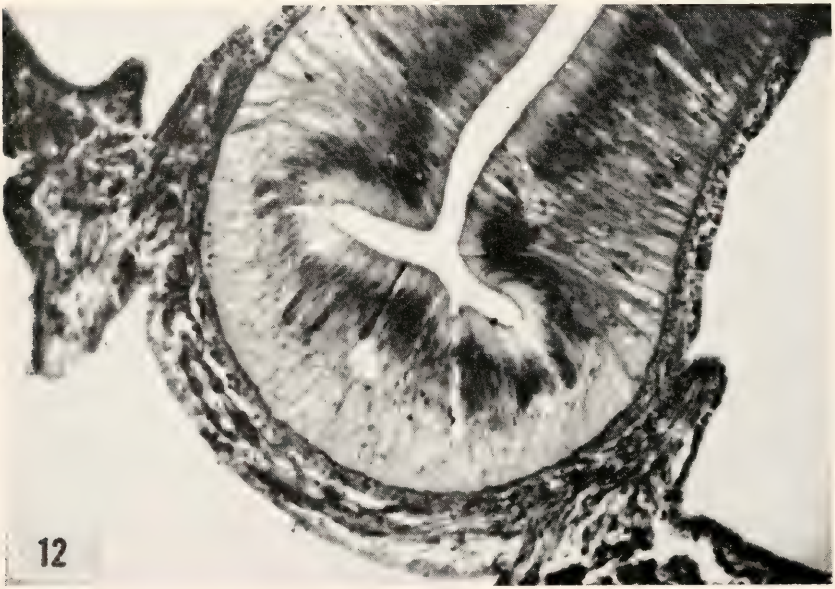

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FIGURE 10. An interradial pouch area, showing abundance of zymogen cells; note also the scanty nature of brush border development and flagella. PTAH; 235 ×.

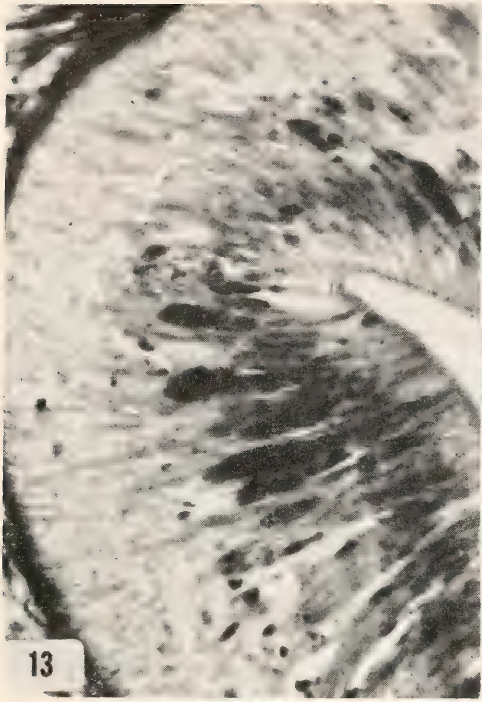
cells and enter the roots that attach to the surface of the underlying connective-tissue layer. The apical ends of typical cells are provided with a brush border, sometimes rather scanty, about the base of the flagellum. Variations in such typical cells, and in the distribution of different types of secretory cells among them, form the basis of the marked regional specializations characteristic of the digestive tract in *Henricia.*

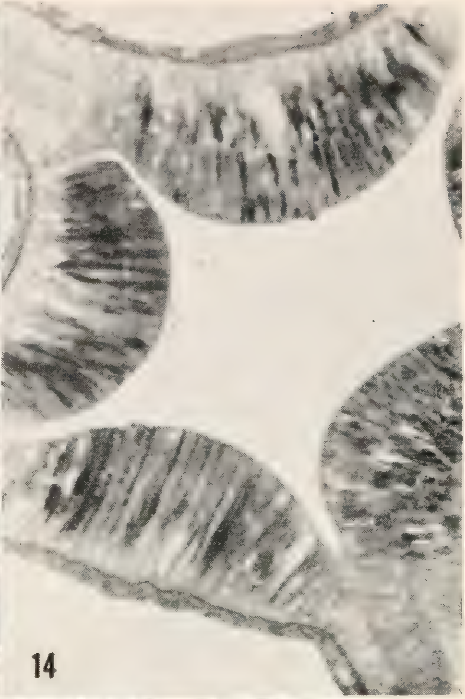
At the extreme oral end of the cardiac stomach, in the region termed "esophagus" by Hayashi and others, the epithelium lies in numerous folds, supported by lamellar inward extensions from the basal connective-tissue layer. Scattered among the typical epithelial cells here are occasional clumps or strings of secretory granules, and somewhat more numerous tall, flask-shaped mucous goblets with distorted, deeply staining basal nuclei. These features are brought out by staining with phosphotungstic acid hematoxylin (PTAH) and are illustrated in Figure 6. By far the most conspicuous aspect of the epithelium in this region, however, concerns the clear and empty appearance of the apical ends in all of the otherwise typical cells here; PTAH stains nothing (other than flagellary basal bodies) in the distal quarter or so of these cells. In contrast (Fig. 7), treatment of adjacent sections with dilute toluidine blue elicits a most vivid gamma metachromasia in precisely those parts of the cells that fail to stain with PTAH. The same regions are stained by Harris' hematoxylin, selectively colored by Alcian Blue, and even after salivary digestion give a positive reaction with the periodic-acid-Schiff technic. Such staining behavior indicates that the apical portions of the typical cells in this portion of the stomach are filled with masses of an acid mucopolysaccharide, and this is the only place in the entire digestive tract where such materials are found to be so intimately and so copiously associated with "typical" epithelial cells. Metachromatic staining, along with the other reactions described for these cells, is also exhibited by the globular or flocculent contents of the mucous goblets, by material associated with the brush border, and by numerous rather coarse granules or deposits randomly scattered in the deeper parts of the epithelial cells (Fig. 7).

Above this region the metachromatic staining of the typical cells gradually disappears, except for that associated with the brush border, and the wall of the stomach differentiates into its radial and interradial pouches. A clear distinction can usually be made between these two series of pouches on the basis of their epithelial characteristics. In the relatively narrow and deep radial pouches, the epithelium consists almost entirely of typical cells, very crowded, with conspicuous, long flagella and well-developed apical brush borders (Figs. 8, 9); in these areas there are few secretory cells, but some zymogen cells are present and some mucous cells containing flocculent secretion. By contrast, the larger and broader interradial pouch areas exhibit an epithelium very rich in mucous goblets and with relatively large numbers of zymogen cells (Figs. 10, 11). Although the typical

FIGURE 11. Mucous gland cells in an interradial pouch area comparable with that shown in Figure 10. Periodic-acid-Schiff after salivary digestion, Weigert hematoxylin, fast green. Note the tall, slender, deeply-staining mucous goblets, and the diffuse positive stain in the distal parts of the epithelium after removal of all glycogen. 470 ×.

FIGURE 12. Radial gutter in cardiac stomach, upward extension of a radial pouch. Tall, crowded, conspicuously-flagellated cells, well-developed nerve plexus layer; note also the large, darkly-stained mucous goblet. PTAH; 235 ×.





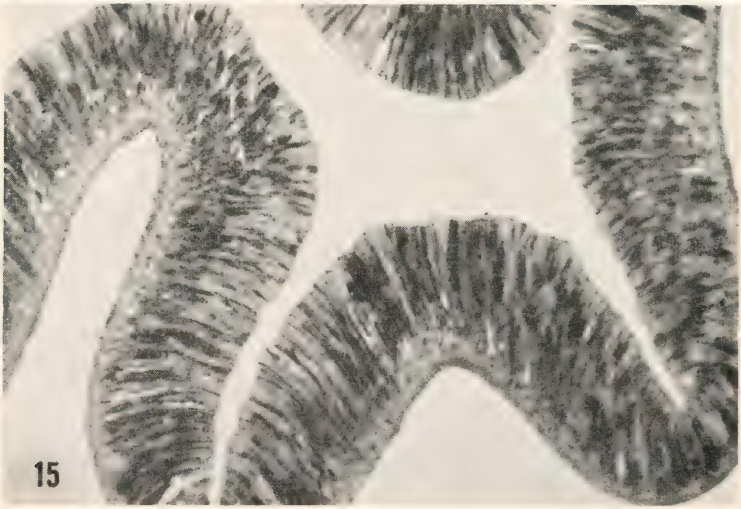


FIGURE 13. Detail of a region similar to that shown in Figure 12. Note bulbous mucous goblets, with scattered spindle-shaped bodies deep in the epithelium (*cf.* Fig. 16). PTAH; 470 ×.

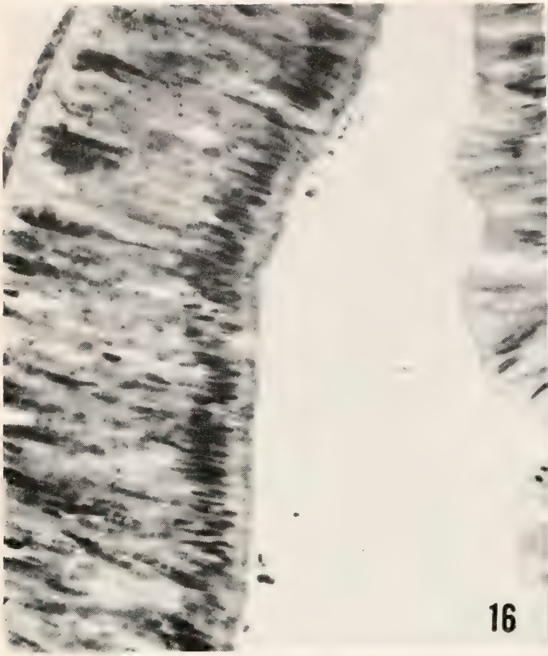
FIGURE 14. Frontal section through the central aperture leading into the pyloric stomach, showing secretory type of epithelium with numerous mucous glands, scanty brush borders and flagella. PAS-Weigert-fast green, salivary digestion; 235 ×.

FIGURE 15. Frontal section through portion of radial reservoir of the pyloric stomach, stained to reveal the abundance of zymogen cells in this region. PTAH; 235 ×.

cells that are found here show flagella on close examination, these are sparse and relatively short, and the brush borders are scanty. These general distinctions in epithelial characteristics follow consistently upward through the cardiac stomach; the crowded, flagellated, conspicuously brush-bordered cells of the radial pouches continue upward through the radial gutters, crossing into the pyloric stomach as tracts leading to the marginal openings in its walls, with the addition of a large, dense, bulbous type of mucous gland cell (Figs. 12, 13). The richly secretory epithelium of the interradial pouch areas, with its short, scanty flagella and brush borders, extends to clothe the centrally-directed vesicles of the cardiac stomach and even to furnish the characteristic lining of the floor and walls of the branching pyloric stomach (Fig. 14). Zymogen cells are numerous throughout, but particularly in the radial reservoirs (Fig. 15). All of these areas also contain very abundant mucous cells, varying from tall, slender goblets with fine granular secretion to large, flask-shaped glands filled with coarse, homogeneous globules, resembling those encountered in the radial gutters. These may represent different secretory phases of a single basic type of mucous gland. In the central folds between radial branches of the pyloric stomach the epithelium exhibits an interesting feature; interspersed among the upper ends of the tall, crowded epithelial cells lie small, spindle-shaped bodies staining deeply with the hematoxylins or with carmalum. They increase in abundance in the folds of the radial reservoirs and are particularly numerous and conspicuous in areas where opposite side walls are closely apposed (Figs. 16, 17a, 17b). They are, at least in these areas, almost entirely limited to the upper third or quarter of the epithelium and so are in reasonable proximity to the lumen. These elements resemble most strongly the bodies described by Smith (1937) as primary sense-cells in *Marthasterias;* they are very much like the sense cells Smith found in the radial nerve cord and bear a less striking resemblance to the cigar-shaped nuclei of the cells he interprets as representing the same elements in the lining of the stomach (see also Anderson, 1954). The deeply-staining spindles in my preparations of *Henricia* are evidently nuclei, sometimes granular but most frequently dense and homogeneous. The cytoplasm to which they belong cannot be distinguished, crowded as these bodies are in the great profusion of secretory cells (Figs. 17a, 17b); it probably consists, as Smith's Figure 5 suggests (1937, p. 123), of slender, fibrous processes running downward from the free border of the epithelium into the nerve plexus layer below. Diligent search for similar bodies in the cardiac stomach reveals a number of spindle-shaped nuclei deep in the epithelium of the flagellated tracts, usually in association with large mucous glands (Fig. 13).

In each radial reservoir, an abrupt line of transition is evident where the richly secretory side-wall epithelium gives way to the conspicuously-flagellated typical-cell epithelium (Fig. 18) that covers the roof of the pyloric stomach and continues as the lining of the intestine.

The "granulated gland cells well-stained by eosin" mentioned by Hayashi (1935, p. 10) as a component of the epithelium in the cardiac stomach, and as being present in suddenly increasing numbers in the pyloric stomach, cannot be identified, unless they correspond to what I have called zymogen cells. In my preparations the zymogen granules stain neither with eosin nor with Harris' hematoxylin; they appear as clear, refractile spherules crowded in an eosinophilic ground cytoplasm. This, it will be noted, may offer an answer to the puzzling



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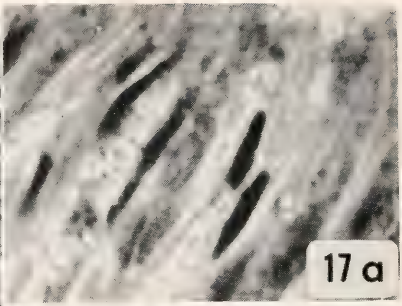

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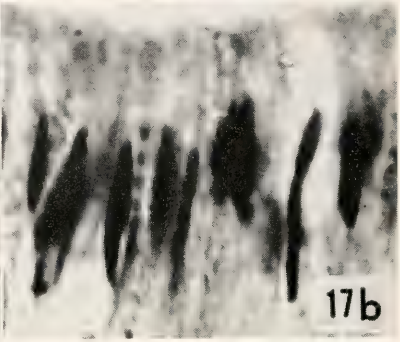

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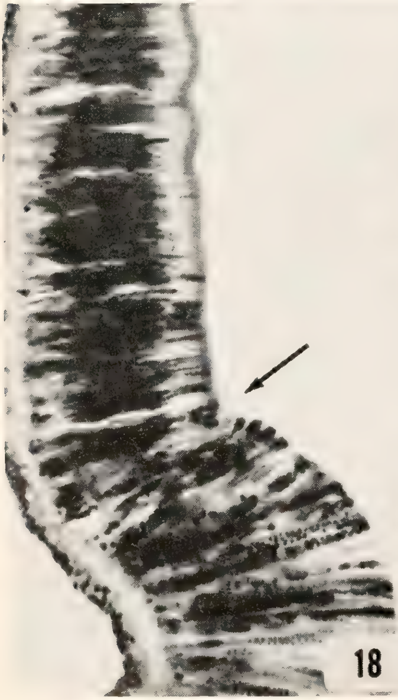

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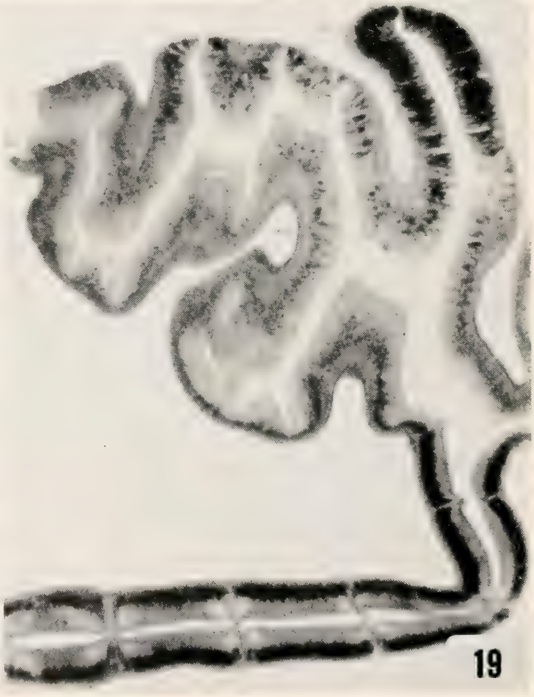

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Figure 16. Side wall of a radial reservoir, showing abundance and localization of spindle-shaped nuclei interpreted as pertaining to sensory cells. PTAH; 470 ×.

problem: the *cells*, as Hayashi says, are granulated and do stain well with eosin; but the granules themselves are not eosinophilic.

The duality so evident in the gross anatomy of the pyloric caecum-Tiedemann pouch complex is revealed also in the histology of the organs. The caecum and its median duct share epithelial characteristics with the central pyloric stomach and its radial reservoirs, while the histological affinities of the pouch are, not surprisingly, with the lower part of the pyloric stomach and even with the radial pouches and gutters of the cardiac stomach. It will be recalled that these areas form continuous flagellated tracts in the stomach, leading directly into the openings forming the roots of the paired Tiedemann pouches. Figure 19 illustrates the striking histological contrast between the upper and lower parts of the caecum-pouch complex.

As each radial reservoir of the pyloric stomach tapers to form the median duct of its caecum, the proportion of zymogen cells in its epithelium increases to a maximum. The roof and side-walls of this median duct present a greater concentration of zymogen cells than any other part of the caecum; the folded walls of the lateral diverticula branching from the duct always contain large numbers of such cells (Figs. 19, 20), but nowhere in these lateral areas do they occur in such profusion as in the median duct. In the scattered zymogen cells of the diverticula, but not in the crowded ones of the duct, the masses or strings of granules are commonly accompanied by clear vacuoles (Fig. 21), a condition previously noted in the caecal zymogen cells of *Asterias forbesi* (Anderson, 1953) and still without explanation. The epithelium of the median duct, and to a lesser extent that of the lateral diverticula, also contains numerous mucous goblets like those found in the pyloric stomach, as well as a considerable representation of the dense spindle-shaped bodies interpreted as pertaining to sense-cells. These last are very difficult to find in the lateral diverticula and particularly so in the outer parts.

Beyond these special features, the pyloric caeca present little to distinguish them histologically from those of *Asterias forbesi* (Anderson, 1953). Most of the cells making up the epithelium of the lateral diverticula are relatively undifferentiated, with flagella and apical brush borders, and evidently function chiefly as absorptive and storage cells. Most of them contain several to many coarse, granular bodies, usually lying below the level of the nucleus (Fig. 21). These are highly basophilic and are Schiff-positive after periodic acid oxidation, resisting salivary digestion; they are not metachromatic and do not stain with Alcian Blue. Although this is insufficient for specific characterization, the staining behavior of these bodies suggests that they consist of some type of mucopolysaccharide that may be serving as a nutritional reserve. In contrast to the storage cells in the caeca of *Asterias*, those of *Henricia* appear to contain very little glycogen

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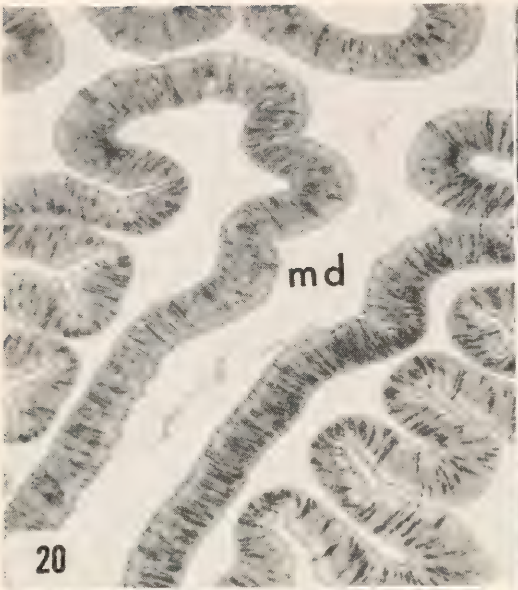
FIGURES 17a, 17b. Details of sense-cell nuclei. PTAH; 1100 ×.

FIGURE 18. Sharp transition zone (arrow) between richly secretory side-wall epithelium of radial reservoir (*below*) and current-producing, flagellary epithelium of roof (*above*). PTAH; 470 ×.

FIGURE 19. Overall cross-section of a caecum-pouch complex; the pouch (*below*) normally hangs straight down (*cf.* Fig. 5) but has been bent over in histological processing. Note the marked localization of deeply-staining zymogen cells in the median caecal duct (*upper right*), and the arrangement of the parallel flagellated channels traversing the pouch from the gutter (*lower left*) to the lower part of the median duct. Seam-cell adhesions separate adjacent channels. PTAH; approximately 75 ×.



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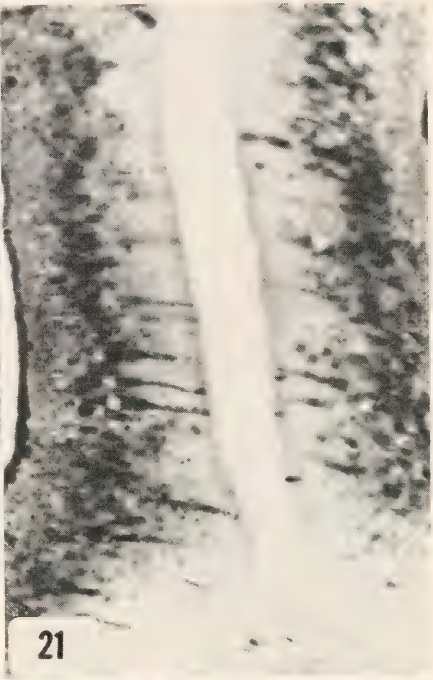

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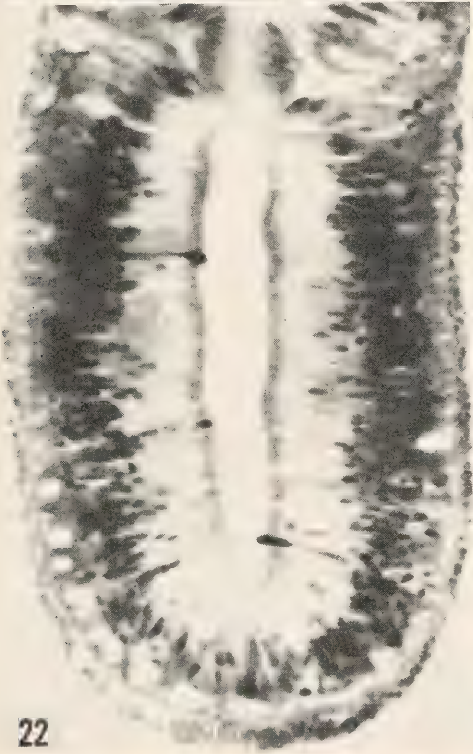

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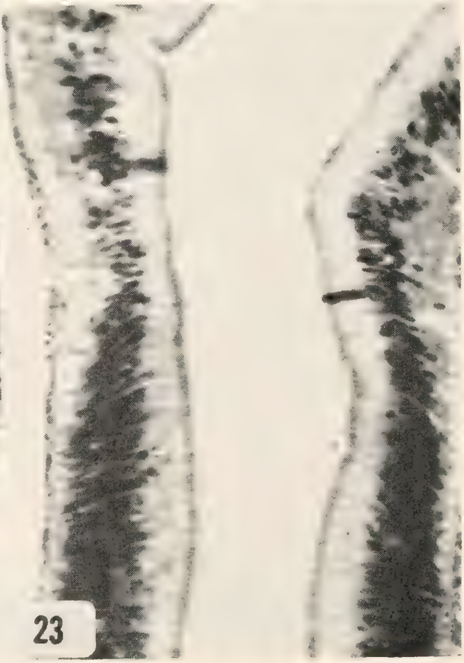

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FIGURE 20. Frontal section through side wall of median duct, showing relationship between its lumen (*md*) and the lateral diverticula branching from it. Note also distribution of zymogen cells (*cf.* Fig. 19). PTAH; approximately 75 ×.

or lipid. This condition is perhaps related to the fact that my specimens of *Henricia leviuscula*, from which tissues were taken for the periodic-acid-Schiff and Sudan black technics, were collected just as they entered their breeding season. Although detailed studies involving *Henricia* have not been made, the work of Greenfield, Giese, Farmanfarmaian and Boolootian (1958) has conclusively demonstrated for two other starfishes that glycogen and particularly lipids are transferred from storage in the caeca for utilization in the ripening gonads as the breeding season approaches.

Along a line at the lower edge of the median duct, below the origins of the lateral diverticula, the epithelium changes abruptly (Figs. 19, 23). This line marks the junction between Tiedemann's pouch and the caecum proper. Within the pouch, the epithelium of the side walls is almost a pure population of "typical cells"; zymogen cells are few and widely scattered, and mucous cells, as will be seen, are strictly localized according to a definite pattern. The typical cells are extremely crowded and laterally compressed; the nuclei lie in a wide band of varying levels, a brush border is well developed, and each cell bears one or two (difficult to determine) long and powerful flagella, with conspicuous, elongate basal bodies and intracellular fibrils.

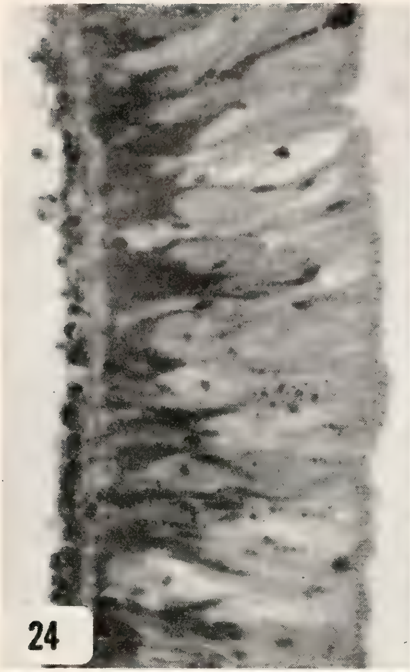
The floor of Tiedemann's pouch is narrow, formed by a gutter leading outward from one of the marginal openings low in the pyloric stomach. The epithelium lining this gutter contains an abundance of large mucous gland cells of the dense, bulbous type, their swollen cavities packed with globular secretion masses distorting their own nuclei and crowding the neighboring epithelial cells in all directions (Figs. 22, 24). Deep in the epithelium, between the mucous goblets, lie a considerable number of spindles, like those seen in similar locations in the cardiac stomach.

At regular intervals, bands of the crowded mucous-epithelial cells pass diagonally upward from the gutter to the lower margin of the caecum above, traversing the normal epithelium of the side walls of the pouch as a series of parallel striations clearly visible through the wall of the organ (see Figure 5). A transverse section of the caecum-pouch complex, such as that shown in Figure 19, cuts across several of these striations; examination reveals that each is a line of adhesion between corresponding bands of mucous-epithelial cells on facing walls of the pouch. These seams divide the pouch into approximately 30 parallel channels (in an average-sized animal), running diagonally upward from the gutter to join the median duct of the caecum, each channel lined on both its side walls by a type of epithelium evidently highly specialized for the production of powerful flagellary currents. The seams or lines of adhesion separating adjacent channels involve bands of tissue several to many cells wide, in which the nuclei in both of

FIGURE 21. Detail of epithelium in a lateral diverticulum. Note vacuoles associated with zymogen granules, and the abundant coarse granules below the nuclear level, interpreted as mucopolysaccharide deposits. PTAH; 470 ×.

FIGURE 22. Cross-section of the oral gutter of Tiedemann's pouch, bounded at top by an adhesion-seam. Note the crowded current-producing cells in the side wall, the few and scattered zymogen cells, the abundant mucous gland cells in the floor of the gutter (*cf.* Fig. 24), and the thickened tracts (clear basal circles) of the nerve plexus layer in the floor of the gutter. PTAH; 470 ×.

FIGURE 23. Transition zone marking the junction between Tiedemann's pouch and the pyloric caecum, where the current-producing typical-cell epithelium of the pouch gives way to the secretory and storage-cell epithelium of the caecum. PTAH; 470 ×.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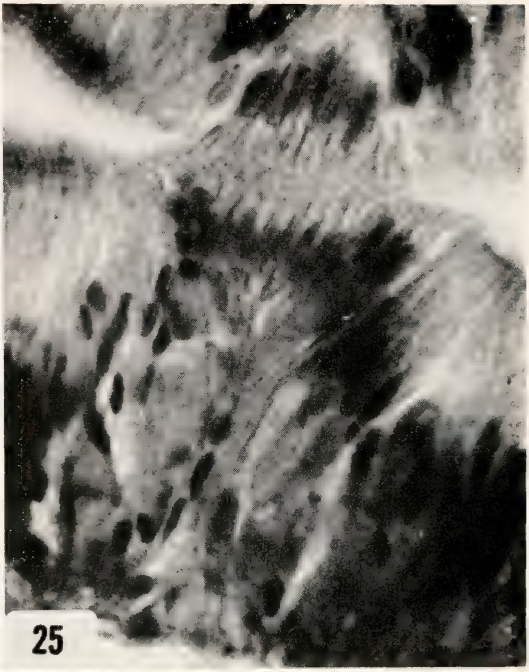
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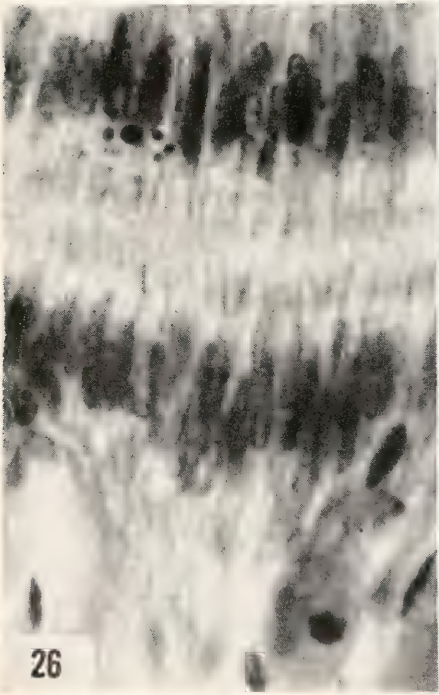
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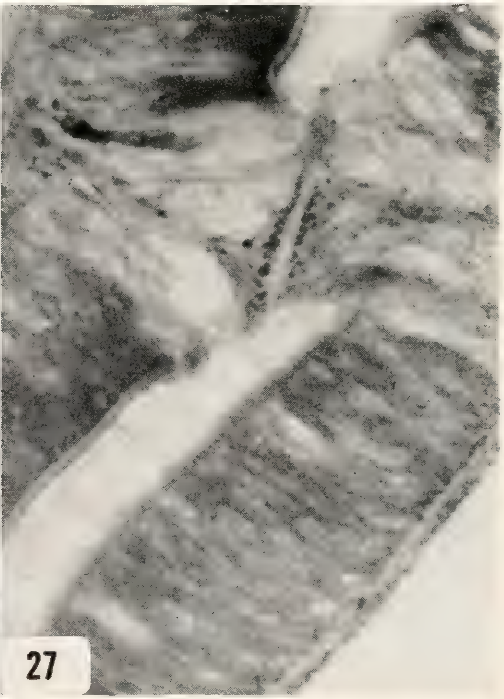
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FIGURE 24. Frontal section, side wall of oral gutter in Tiedemann's pouch, to show the abundance of huge, bulbous mucous glands characteristic of this area. Note also spindle-shaped nuclei scattered deep in the epithelium. PTAH; 470 ×.

the apposed epithelia lie exceptionally high in the tissue. In section (Fig. 25) they give the impression of having been heaved or herniated outward from the basement membrane by pressure from the swelling bands of mucous gland cells between which they are confined.

The localization of the herniated seam-cells and their flanking bands of mucous cells, and the regular alternation of these zones of adhesion and the flagellated channels which they separate, are clearly shown in Figure 19. Although their nuclei have been raised almost to the level of the free edge of the epithelium, the seam-cells retain their attachment to the basal connective-tissue layer by way of elongated, compressed cytoplasmic stalks in which supporting fibrils are strongly developed. These intracellular fibrils continue upward past the nuclear level and form a characteristic and conspicuous feature of the distal ends of the joined cells (Figs. 25, 26). They do not, however, cross the plane of fusion to join the fibrils of cells in the opposite walls, and I have seen no other evidence of actual cytoplasmic continuity between the adherent cells; in favorable preparations a distinct double membrane can be observed at the seam-line. Nevertheless, the adhesion-seams are firm, close, and evidently permanent; when the tissue is broken or torn at a seam in the course of histological manipulations, it is noteworthy that the seam almost never tears at the line of fusion but rather just below the nuclei of the seam-cells of one side or the other.

The distal ends of the seam-cells contain a number of moderately coarse granular deposits which in their staining behavior resemble the Schiff-positive globules of the storage cells in the pyloric caecum. As in the caecum, very little glycogen or lipid appears in any of the cells of Tiedemann's pouch. Oddly, the only conspicuous sudanophile inclusions in the pouch areas lie in the distal ends of the seam-cells, appearing as discrete droplets scattered in the supranuclear cytoplasm (Fig. 27). The significance of lipid localization in these highly specialized and aberrant cells is problematic.

The sense-cell spindles identified deep in the epithelium of the gutter accompany the adhesion-bands also. Here they lie among the swollen mucous goblets (Fig. 25) and are often compressed against the elongate, fibrous stalks of the seam-cells. Smith has noted that in *Marthasterias* the sense-cells occur principally in those areas of the gut where the subepithelial nerve plexus layer is particularly well developed. In this connection it is of interest that in *Henricia* the floor of the gutter is underlain by several notably thickened tracts of the plexus layer (Fig. 22), and that similar thickenings accompany the specialized adhesion-tracts and unite with conspicuously well-developed areas of the plexus layer along the line of attachment between Tiedemann's pouch and the caecum (Fig. 23). The correspondence in distribution between the thickenings of the plexus layer and the

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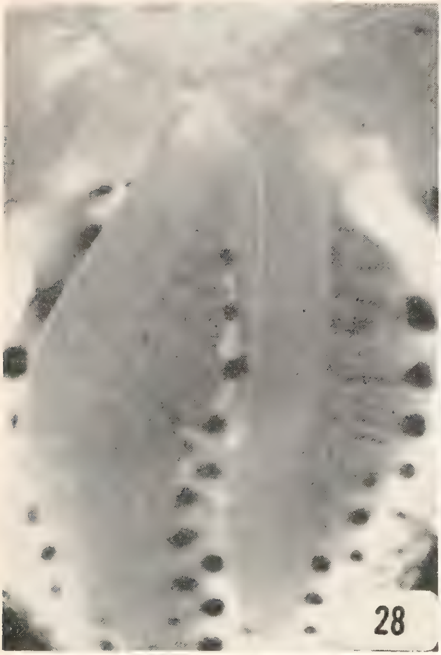
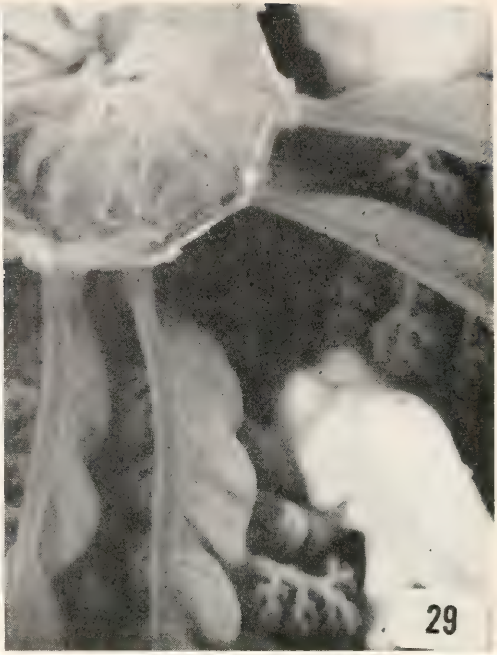
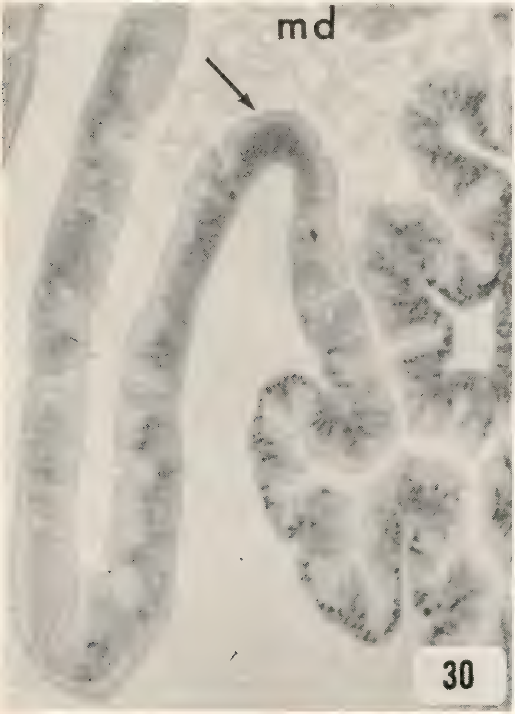
FIGURE 25. Detail of a seam-cell adhesion-band in Tiedemann's pouch. Note the apparent herniation of the seam-cell nuclei upward, and the attachment of these cells to those opposite in the epithelium across the lumen. The seam-cells maintain attachment to the basal connective-tissue layer; note the band of bulbous mucous goblets flanking the seam-cell zone on both sides, scattered sense-cell nuclei, and crowded typical-cell epithelium in channels to left and right. PTAH; 1100 ×.

FIGURE 26. Distal ends of apposed seam-cells. Note that the intracellular fibrils are not continuous across the line of fusion. PTAH; 2100 ×.

FIGURE 27. Sudanophilic droplets in distal ends of seam-cells. Sudan black-carmalum; 470 ×.







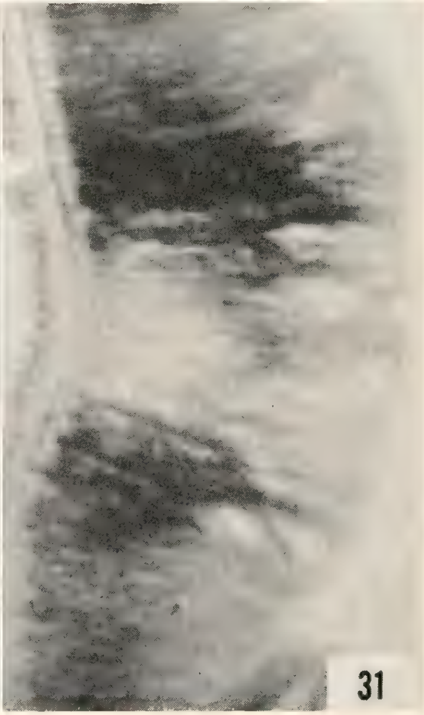


FIGURE 28. A pair of caecum-pouch units in *Patiria,* from below. Note the separate origins from the pyloric stomach, and the broad, bag-like nature of the Tiedemann's pouches. Dissection of a living specimen, approximately 5 ×.

concentrations of sense-cell spindles in Tiedemann's pouch seems unlikely to be merely coincidental.

C. Function

The relatively small size of the cardiac stomach, and the absence of voluminous gastric pouches, make it appear improbable that feeding in *Henricia* involves eversion of the stomach to the extent characteristic of *Asterias* or *Patiria*. Living specimens at rest are frequently observed with the mouth widely open, with folds of the lower cardiac stomach lying over the peristomial lips. Within the stomach the lumen is largely occupied by the swelling vesicles of the upper cardiac stomach, framing the narrow, stellate opening into the pyloric stomach. In some specimens, these vesicles appear partially everted through the mouth, where they form lobe-like structures extending only about a millimeter. It is to be noted that the stomach is provided with a stout muscular and fibrous retractor harness which, while differing markedly from that described for *Asterias* and *Patiria* (Anderson, 1954, 1959), still appears much too well-developed to be merely an anchor for the resting stomach. The action of the stomach suggests that the retractors function in securing the vesicular lobes, adjusting their degree of eversion, and maintaining the folds or gutters between them.

The organization of the various flagellated tracts, ducts, and channels suggests that the digestive tract in *Henricia* is particularly well adapted for the production of currents and the transport of suspended particles. The existence of such currents, and their intricate interrelationships within the digestive tract, can be demonstrated by the use of suspensions of India ink or stained yeast cells. Particles move rapidly upward through the radial pouches and gutters of the cardiac stomach, into the pyloric stomach, and out through the marginal openings into Tiedemann's ducts. In dissected specimens, suspensions introduced into one of these ducts are observed to pass distally with astonishing rapidity through the gutter of the pouch, from which successive streams are diverted and directed upward through all the diagonal flagellated channels into the median duct of the pyloric caecum above. Reaching this, particles move into the lateral diverticula of the caecum, up their side walls, and back across their roofs into the aboral part of the median duct. Here, a strong centrally-directed current streams inward, moving the suspension across the folded surfaces of the radial reservoir and into the narrow central lumen of the pyloric stomach. Currents converging here from all the radial reservoirs pass downward into the lower part of the pyloric stomach, where particles again come under the influence of the radial flagellated gutters and the currents setting towards the marginal openings and into Tiedemann's

FIGURE 29. Detail of similar structures. Note the oral gutter on the margin of Tiedemann's pouch, and the fold-patterns traversing the side walls. Approximately 10 ×.

FIGURE 30. Cross-section of a caecum-pouch complex. Note the absence of flagellated channels or adhesion seams, the relationship between the broad lumen of the pouch and the median duct (*md*) of the caecum above, and the crowded band of typical cells (*arrow*) at the junction between pouch and caecum. Zymogen cells are also visible in the epithelium of the lateral diverticula. PTAH; 75 ×.

FIGURE 31. Sudanophilia in side-wall epithelium of Tiedemann's pouch, *Patiria*. Note that the mucous-gland patch (clear center area) is flanked on each side by a band of highly sudanophilic cells which gradually fades into a typical-cell epithelium. These alternating bands are responsible for the appearance of parallel folds in the side walls. Sudan black-carmalum; 470 ×.

ducts. A complete and very active circulation through the digestive tract is thus maintained, featuring one-way transport of solutions or suspensions by specifically-directed flagellary currents. Sorting mechanisms undoubtedly occur; in the radial reservoirs, for example, selected particles are probably carried across the roof of the pyloric stomach and into the intestine for elimination. But it is clear that a given particle, until thus eliminated or until completely digested, can make innumerable circuits through the secretory regions of the digestive system. There are regional differences in transport velocity—currents in the lateral diverticula of the caeca, for instance, move much more slowly than those in Tiedemann's ducts and pouches—but there appear to be no static areas and no chambers in which large masses of ingested food can lie while undergoing dissolution.

D. Tiedemann's pouches in other starfishes

As pointed out by Cuénot (1887), members of the Family Echinasteridae (*Echinaster, Henricia*) share with those of the Family Asterinidae (*Asterina, Patiria*) conspicuous development of the organs we are calling Tiedemann's pouches. In *Patiria miniata,* for example, the paired caeca of a single ray originate separately from the pyloric stomach, and a well-developed, elongate pouch hangs from the oral midline of each caecum (Figs. 28, 29). The lower margin of the pouch is formed by a tubular gutter, taking its origin, like the similar structure in *Henricia,* from an opening in the wall of the stomach just above the circumferential fibrous girdle. As in *Henricia* also, the walls of the pouch are traversed by a regular series of parallel folds, visible externally, that run diagonally upward from the gutter to the median duct of the pyloric caecum above. The lateral diverticula of the caecum form complexly folded pockets arising at intervals from the lateral-aboral walls of the median duct. Although superficially similar, the caecum-pouch complexes in *Patiria* and *Henricia* present many fundamental differences. *Patiria* lacks, for example, the specialized portions of the pyloric stomach termed radial reservoirs in *Henricia;* Tiedemann's pouch and the median duct of the caecum open into the pyloric stomach by a common aperture, tall and narrow but not divided. Histological comparison reveals further contrasts (Fig. 30). Within the caecum, zymogen cells, mucous gland cells, and storage cells are more or less uniformly distributed, without the marked segregation of zymogen cells in the median duct so conspicuous in *Henricia.* It will be noted also that Tiedemann's pouch is more broadly attached below the caecum, and that the pouch itself is broader and more bag-like. This is explained by the fact that, as Figure 30 shows, the opposite walls of the pouch are not held together by mucous-epithelial adhesions as in *Henricia* but are only approximately parallel and not closely apposed. The conspicuous striations in the walls, superficially so similar to those of *Henricia,* are produced by parallel bands of large, crowded mucous gland cells alternating with bands of extremely crowded, very tall "typical" cells. A notable concentration of these non-secretory cells is always found in the zone of attachment where Tiedemann's pouch joins the median duct of the caecum. No zymogen cells are found in the pouch. The distribution of lipids is significantly different from that in the pouch of *Henricia.* All of the crowded typical cells show accumulations of sudanophile droplets in their basal portions, even extending through the thin strands that penetrate the thick nerve plexus layer and insert on the connective-tissue sheet. (Fig. 31). The mucous gland cells contain no notable lipid deposits, but each of the mucous bands is flanked by strips in which the typical cells contain exceptionally high concentrations of lipid droplets. These fade gradually into the normal typical cells in the areas between the mucous bands. There are no cells here comparable to the herniated seam-cells of *Henricia* with their apical concentrations of sudanophile droplets.

Suspensions of stained yeast cells introduced at the opening leading into one of the pouches are carried through a pattern of circulation very similar to that observed in *Henricia*. Even though the walls of the pouch do not adhere to form separate channels, the concentrated flagellary bands are capable of producing rapid currents that distribute materials along the entire length of the median duct. There are no quiet or stagnant areas; whatever enters the pouch moves immediately into the caecum, then back to the pyloric stomach and outward through the pouch again. The general pattern described by Irving (1924) in the caecum-pouch complex is verified, although the anatomical relationships shown in his figure (p. 117) are inaccurate.

Without going into detail, brief anatomical and histological examination of the digestive tract of *Asterina gibbosa* shows that the features of its pyloric caecum-Tiedemann pouch complex are very similar to those of its close relative *Patiria* and differ in the same ways from those of *Henricia*.

A figure sketched by Richters (1912) in his study of regeneration in *Linckia* led me to suspect that the pyloric caeca of this starfish might also bear Tiedemann's pouches, although this has not been reported in the literature and *Linckia* is placed in a different order (Phanerozonia) from that to which the asterinids and echinasterids belong (Spinulosa) (see Hyman, 1955, pp. 334, 336–337). Preliminary studies on preserved specimens of *Linckia guildingi* reveal that Tiedemann's pouches are indeed present and are larger and better-developed than those of any other species examined so far. They extend almost to the distal ends of the long pyloric caeca, and their proximal ducts are separated by partitions from specialized portions of the pyloric stomach resembling the radial reservoirs of *Henricia*. Transverse sections of the pouches show that adhesion-bands similar to those of *Henricia* divide the pouch into separate flagellated channels. The gutter forming the oral margin of the pouch is divided by a fold arising from its floor, which externally encloses a stout band of muscle fibers. Altogether, although details have not been exhaustively studied, it is evident that the caecum-pouch complex in *Linckia* is strikingly similar in its fundamental characteristics to that described in *Henricia*. This is the more interesting in view of the wide taxonomic gap that apparently separates the two genera.

Astropecten, in which Tiedemann (1816) originally described the pouches that now bear his name, has very small ones extending a centimeter or less along the oral side of the median duct of the caecum. Their side walls show a few moderately developed parallel striations, but in the preserved material on which I have made a preliminary study these do not form firm adhesions across the lumen.

DISCUSSION

There are many anatomical and histological differences between the organs of the digestive tract in *Henricia* and those of other starfishes in which details have been described. The small size of the cardiac portion of the stomach, the large size and peculiarly branched structure of the pyloric portion, the conspicuous development of the rectal caeca, and other features, were noted by Hayashi (1935). Further, the cardiac stomach of *Henricia* lacks any hint of the elaborately branching patterns of flagellated gutters, with accompanying ramifications of the retractor strands and corresponding localizations of specialized epithelial cells, characteristic of the cardiac stomach in *Asterias, Pisaster, Pycnopodia,* and *Patiria* (Anderson, 1954, 1959). Lacking these, the stomach of *Henricia* is not, however, without specializations of its own, such as the so-called esophageal pouches and the alternating gutters and vesicles at higher levels, with their marked histological distinctions. Other histological peculiarities are common in various parts of the digestive tract. For instance, areas in the cardiac stomach and almost the whole of the pyloric stomach are lined with zymogen cells; in contrast, the zymogen cells of *Asterias forbesi* and *Patiria miniata* are found only in the pyloric caeca. Within the caeca of *Henricia,* the startling concentrations of zymogen cells in the walls of the median ducts are unique; in *Asterias,* this area is given over to a population of tall, crowded, current-producing cells interspersed with mucous goblets, and the zymogen cells are restricted to the lateral diverticula.

Such instances of structural contrast could be multiplied, all leading to the conclusion that in starfishes, as in other animals, corresponding parts of the digestive system, clearly homologous, may be adapted to diverse functions in relation to differences in food and in feeding methods. The food habits and feeding mechanisms of *Asterias* and similar forms are reasonably well understood; that they are probably not at all like those of *Henricia,* which are unknown, is indicated by the conspicuous structural differences revealed by detailed study.

In general, the specializations noted in *Henricia* seem to involve a tendency to segregate tissues with predominantly secretory functions in areas more or less distinct from those serving primarily for current production. Further, while the transport of materials through the gut by flagellary currents is common to all starfishes, in *Henricia* this function has been raised to a peak of efficiency by the development of the special modifications of the pyloric stomach called Tiedemann's pouches. By means of these elaborate organs, suspensions or solutions brought to them by the flagellary tracts of the stomach are very rapidly distributed to the secretory and absorptive areas of the pyloric caeca and pyloric stomach, and maintained in repeated circulation through these areas until digested, absorbed, or eliminated.

Studies elucidating the current-patterns maintained within the digestive tract have been made on a variety of asteroids, including *Asterias rubens, Solaster papposus, Porania pulvillus,* and *Astropecten irregularis* (by Gemmill, 1915); *Patiria miniata* (by Irving, 1924); and *Asterias forbesi* (by Budington, 1942). It is noteworthy that in all of these species, of diverse taxonomic affinities, the patterns are reasonably similar in general outline, with minor variations related to anatomical differences. In all, currents flow in the oral-aboral direction from cardiac to pyloric stomach; radially across the floor of the pyloric stomach and outward along the oral sides of the pyloric ducts, continuing on the oral side of the median duct in each caecum; in a circular fashion through the lateral diverticula and back to the median duct, this time aborally; and finally returning toward the pyloric stomach again in the aboral part of this duct. In the Asteriidae, particularly, the pyloric duct is a restricted passage; within it, however, currents must be maintained both centrifugally and centripetally, the one current running on its oral side, the other on its aboral side.

It is obvious that the feeding and digestive activities of the predatory, carnivorous starfishes in this family place no demands upon their transport mechanisms that are not satisfied by the system as it exists. Yet, if one speculates upon ways in which this system might have developed to operate more efficiently for the maintenance of directed currents in the standard pattern, one is likely to be struck first by the existence of the bottle-neck at the pyloric duct. For increased efficiency, the pyloric duct should be larger, it would seem, or at least taller, so that the centrifugal and centripetal streams might be more widely separated. Alternatively, a continuous partition should be provided, set horizontally between the floor and the roof of the duct, for complete isolation of the two currents. In effect, it will be noted, the pyloric ducts of *Patiria* embody the former principle, while those of *Henricia* follow the latter. These considerations seem to justify the conclusion that in both *Patiria* and *Henricia* the functions of the digestive system have placed a higher premium upon efficiency of the flagellary transport mechanisms than is the case in *Asterias* and its close relatives.

Below and distal to the partition that divides the pyloric duct in *Henricia*, Tiedemann's pouch has evolved into a hydrodynamic organ or flagellary pump of prodigious effectiveness. Its design involves close-set, strongly-flagellated side walls, strengthened, reinforced, and held at what we may assume to be an optimal distance from one another by the unique mucous-epithelial seams, which also break up the otherwise broad epithelial expanse into channels set in regular parallel array. These features make it possible for the organ to develop what must be a considerable suction, as the force developed in all of the 30 or so flagellated channels is transmitted to the upper cardiac and lower pyloric stomach by way of the restricted Tiedemann's duct. Fluids and suspensions are drawn rapidly from the stomach through this duct and are ejected at reduced velocity along almost the entire length of the median caecal duct, into which the channels of Tiedemann's pouch lead. It is reasonable to assume that the development of the 10 Tiedemann's pouches as organs furnishing the principal motive power for circulation through the entire digestive tract brought about the emancipation of areas in the pyloric stomach, pyloric ducts, and median caecal ducts from the primarily flagellary functions they must serve in the Asteriidae, and their development of the richly secretory epithelium that is their chief characteristic in *Henricia*.

In contrast, Tiedemann's pouches in *Patiria* are, as we have seen, broad and bag-like, with no adhesion-seams to hold their side walls in apposition and only their similarities in origin and anatomical relationships to suggest that they are related to the pouches of *Henricia*. The alternating diagonal bands of mucous glands and typical flagellated cells undoubtedly approach in functional significance the parallel channels of *Henricia*, to which they show a superficial resemblance in external appearance. Irving (1924) concluded that Tiedemann's pouches in *Patiria* represent regions specialized for distribution, and all observational evidence bears out this supposition. It is to be doubted, however, that these relatively crudely-developed organs even approach in efficiency the more elaborate ones of *Henricia.* The enlargement of the pyloric duct and the median duct of the caecum resulting from the development of the pouch in *Patiria* must represent an advance over the condition that exists in *Asterias,* but one is led to the conclusion that as organs of distribution, or flagellary pumping organs maintaining circulation within the digestive tract, the pouches of *Henricia* are far superior to those of *Patiria.*[2]

Many features of anatomy and histology in the digestive system of *Henricia*—the small size and doubtful eversibility of the cardiac stomach, the absence of any sizable chambers in which masses of food might lie while undergoing digestion, and the obviously high degree of specialization of mechanisms for maintaining flagellary circulation, among others—suggest most strongly that this animal is dependent in its nutrition upon suspended particulate matter rather than upon predation or any other form of macrophagy. The following experiment was performed as a preliminary test of this hypothesis. Two specimens of *Henricia sanguinolenta,* which had been maintained in an aquarium for 6 weeks, apparently without feeding, were placed in a fingerbowl containing a cloudy suspension of *Mytilus* sperm in sea-water, to which had been added a quantity of minute particles of Nile blue sulfate (sparingly soluble in sea-water). Externally, streams of particles could be observed converging upon the mouth, most rapidly and conspicuously in the ambulacral grooves. The mouths of both specimens remained open, much as in Figure 1, and while coarse clumps or large particles were rejected, smaller particles were either swept directly through the radial peristomial grooves at the angles of the mouth or entangled in strands or sheets of mucus which also moved into the stomach. After 12 hours' exposure to the suspension the animals were carefully washed to remove adherent particles and then dissected for examination of the digestive tract. In both specimens, concentrations of dye particles were found at various places in several of the Tiedemann's pouches.

Such a crude experiment should not be interpreted as having established the fact that in its normal nutrition *Henricia* is dependent upon a flagellary-mucous feeding mechanism or that this suffices for all its needs. The experiment does, however, provide a demonstration that at least under certain conditions particulate matter suspended in the surrounding water finds its way into the digestive tract. Further, it shows that in the intact animal particles so ingested pass into the radial parts of the system by pathways previously deduced on anatomical and histological grounds and on the basis of experiments with excised organs. In the absence of any other information on food and feeding in *Henricia*, all the indirect evidence that can be brought to bear seems consistent with the conclusion that finely-divided particulate matter in suspension, exploited by a flagellary-mucous mechanism, is significant in the nutrition of this starfish. If this is valid, then the very close and detailed structural correspondence between the digestive system of *Henricia* and that of *Linckia*, particularly in relation to the highly specialized Tiedemann's pouches, makes it reasonable to extend this conclusion to *Linckia* also. I can find no information on the food or feeding habits of *Linckia guildingi*.

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[2] I take this opportunity of clearing up a misunderstanding involving functional relationships between the pyloric caeca and Tiedemann's pouches in *Patiria.* Irving (1926) performed digestion experiments with isolated surviving caeca of this species and concluded that these organs are capable of digesting gelatin and absorbing amino acids. Hyman (1955, p. 385) questions this interpretation on the ground that the pyloric caecum of *Patiria* ". . . is underlain on the oral side by an extensive stomach diverticulum (so-called Tiedemann's diverticulum . . .), so that in Irving's experiments the gelatin solution went into this stomach diverticulum and the resulting digestion is probably to be attributed to the stomach." It must be pointed out that in *Patiria* the cavity of the pouch is everywhere completely open to, and in free communication with, the median duct of the caecum, so that anything going into the one is very soon distributed and thoroughly mixed in all parts of the other. The stomach of *Patiria* contains a vanishingly small proportion of zymogen cells, and it has been experimentally established that digestion does not occur in the stomach of an animal that has been deprived of all its caecum-pouch complexes (Anderson, 1959). Zymogen cells do not occur in Tiedemann's pouches in *Patiria,* and the only apparent source of digestive enzymes in this species is in the epithelium of the pyloric caeca themselves. Therefore, while Hyman's criticism was certainly justified in the light of information then available, the facts that have subsequently been established make it clear that Irving's conclusions cannot properly be questioned for the reasons advanced by Hyman.

Flagellary-mucous feeding of the type suggested for *Henricia* (and *Linckia*) is not unknown among starfishes. The experiments and extensive observations of Gemmill (1915) on *Porania pulvillus* demonstrate that alone among the starfishes tested, individuals of this species are capable of maintaining themselves for long periods on a diet of suspended particles only. The external and internal currents by which this diet is manipulated are apparently similar to those described in *Henricia*. Gemmill makes no mention of Tiedemann's pouches in *Porania*, and I have been unable to determine from the literature whether they are present. The powerful currents produced by this starfish suggest the presence of some type of specialization of the general nature of the Tiedemann's pouches in *Henricia*.

Several species of starfishes known to be chiefly macrophagous have been suspected of utilizing particulate matter, also, to some extent. It is interesting that these same forms possess more or less well-developed Tiedemann's pouches. For example, Gemmill (1915) finds that flagellary currents carry considerable amounts of particulate food into the digestive tract of *Astropecten*, a sand-dwelling starfish well known as a voracious carnivore. The behavior of *Patiria miniata*, particularly with regard to its habitual exposure of the huge, everted cardiac stomach in the external environment (Anderson, 1959), strongly suggests that it may in this way collect significant amounts of particulate matter to add to its extremely varied diet. *Astropecten*, it will be recalled, has relatively small Tiedemann's pouches, while those of *Patiria* are much larger but of simple construction compared with the elaborate pouches of *Henricia* and *Linckia*. The significance of these structures may lie in the enhancement of circulatory efficiency which even small or simple pouches may contribute, in relation to feeding currents. The marked structural similarity between the digestive tract of *Patiria* and that of *Asterina gibbosa* (which according to MacBride, 1906, subsists principally upon sponges and ascidians) is strongly suggestive of extensive functional correspondences, perhaps including particulate feeding.

Resemblances involving visceral organs, and particularly those of the digestive tract, are usually considered less reliable as indices of taxonomic relationship than, for example, similarities in skeletal structures or other hard parts. Nevertheless, it is interesting to consider from this standpoint the fragmentary information available concerning the distribution of Tiedemann's pouches in starfishes. The similarities between the pouches of *Patiria* and those of *Asterina* are to be expected in genera belonging to the same family. Although the flagellary pumping organs of the *Patiria* type superficially resemble those of *Henricia* and both are called Tiedemann's pouches, they are fundamentally very different structures. The resemblances are so superficial, and the structural differences so profound, that one is led to conclude that in the Asterinidae and Echinasteridae these organs have evolved independently and convergently as solutions to the problem of increasing circulatory efficiency within the digestive tract. These two families, it may be noted, are by all accounts closely related members of the Order Spinulosa.

In contrast, Tiedemann's pouches and other features of the digestive tract in *Linckia* are astonishingly similar, at least in the material available to me for preliminary study, to those of *Henricia*. The similarities are so striking and extend to such details as to make it appear unlikely that they could have arisen convergently in unrelated lines of starfishes. Yet the Linckiidae, chiefly on the basis of skeletal features that are fundamental in asteroid taxonomy, are placed nowhere near the Echinasteridae but among the valvate Phanerozonia. However, MacBride (1906, p. 471) makes the following statement about the Linckiidae, interesting in the present context: "It is possible that these forms, so different in many respects from the other families of the order, may have been directly derived from the long-armed Echinasteridae." My observations on structural details of the digestive system seem strongly to support the idea of a closer relationship between these families than is indicated by the criteria commonly used in starfish systematics.

It is obvious that any attempt to survey similarities and differences in organ systems, and particularly to trace the distribution of anatomically and histologically specialized visceral structures, among the various families and orders of starfishes will be meaningless until it can be based upon vastly augmented data. These data accumulate slowly, requiring careful and detailed study of the internal anatomy of many species on which the literature to date offers only descriptions of external appearance, skeletal features, geographical distribution, and the like. The present attempt at a comparative study of Tiedemann's pouches in a small group of starfishes is inadequate, based as it is upon insufficient information. At least, however, it provides a tantalizing glimpse of the surprisingly broad areas of knowledge still awaiting exploration and interpretation in the Asteroidea.

Summary

1. Detailed study of the digestive system in *Henricia* reveals that as a result of regional differentiation, particularly involving the lining epithelium, areas specialized for zymogenic and mucous secretion are segregated from other areas adapted for current-production. Secretory areas include the five interradial pouches and vesicles of the cardiac stomach, the pyloric stomach generally but particularly its radial reservoirs, and the median ducts and lateral diverticula of the pyloric caeca. Current-producing areas include the five radial pouches of the cardiac stomach and the gutters leading upward to marginal openings low in the pyloric stomach, and especially the very elaborate Tiedemann's pouches which spring in pairs from these openings and extend along the oral midlines of the pyloric caeca. Other starfishes, such as *Asterias,* which lack Tiedemann's pouches, restrict their zymogen cells to the lateral diverticula of the caeca and crowd current-producing cells into the median caecal ducts, an area which in *Henricia* contains an extremely high concentration of zymogen cells.

2. Tiedemann's pouches in *Henricia* are divided into numerous parallel flagellated channels leading diagonally upward into the pyloric caeca. These channels are separated by unique partitions formed by adhesion-seams between opposite side-walls of the pouch. It is evident from their structure and anatomical relationships, and has been experimentally demonstrated, that Tiedemann's pouches are flagellary pumping organs of great effectiveness. They produce currents capable of drawing suspensions or solutions from the stomach and delivering them rapidly along almost the entire length of the pyloric caeca. Centripetal currents stream back into the stomach, and thus a constant circulation of materials can be maintained through the radial secretory and absorptive areas, depending chiefly upon currents generated in the close-set channels of the ten Tiedemann's pouches.

3. The customary food and the feeding habits of *Henricia* are unknown, but several lines of evidence, anatomical and experimental, combine to suggest that this starfish, like at least one other (*Porania*), may subsist either wholly or in part upon suspended particles gathered by a flagellary-mucous mechanism.

4. In *Patiria miniata,* a species not distantly related to *Henricia* (same order, different family), Tiedemann's pouches are present and lie in about the same relationship to the pyloric caeca but are fundamentally dissimilar; they lack separate flagellated channels, the side walls being traversed only by parallel stripes of mucous cells alternating with bands of typical cells, and thus are more bag-like in general structure. Although they function similarly to those of *Henricia,* these much simpler pouches are probably less effective in current production. *Asterina gibbosa,* closely related to *Patiria,* has *Patiria*-type Tiedemann's pouches. *Astropecten,* in which these pouches were originally described (1816), has relatively small ones of simple construction. Both *Patiria* and *Astropecten* have been suspected of supplementing their macrophagous diet by flagellary-mucous particle-feeding, and although their pouches are far less elaborate than those of *Henricia* they are probably of significance in this connection.

5. It is disconcerting to find that *Linckia,* not at all closely related to *Henricia* (different order), nevertheless has Tiedemann's pouches and other specializations of the digestive system similar in most respects to those of *Henricia.* The differences between the pouches in *Henricia* and *Patiria* are so fundamental as to suggest that they represent independently evolved solutions to the problem of increasing circulatory efficiency within the digestive tract. In contrast, the pouches of *Henricia* and *Linckia* resemble each other so strikingly that it is difficult to conceive of them as having been produced by convergent evolution.

6. It is pointed out that detailed studies on many species of starfishes now known only from external anatomy and skeletal features of preserved specimens will provide information upon which to base broader and more meaningful comparative surveys of internal specializations.

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THE FEEDING BEHAVIOR AND RESPIRATION OF SOME MARINE PLANKTONIC CRUSTACEA[1]

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Interest in the respiratory rate and food requirements of the marine zooplankton probably dates from the studies of Pütter (1907, 1909), but data are presently available for only a dozen or so species of neritic copepods and for *Euphausia pacifica* (Lasker, 1960). *Paraeuchaeta norvegica*, studied by Raymont and Gauld (1951), is the only marine copepod for which respiratory measurements are available, which commonly occurs at depths greater than 100 meters.

In the present work a number of shipboard and laboratory experiments were performed with oceanic species of copepods, amphipods and euphausids, some from deep water, to learn if such organisms were amenable to artificially controlled conditions, and to obtain additional respiratory data for a very important but little-studied group of animals. A few species previously investigated from other localities (*i.e.*, *Calanus finmarchicus*) were also included for comparative purposes.

In addition to the respiratory measurements, representatives of most species investigated were also kept in laboratory culture vessels where observations were made on their behavior and food habits. Some individuals of the copepod, *Calanus hyperboreus*, have been maintained in the laboratory for approximately one year which would seem to be the life span for it. Because of its large size, long life span, and ability to adapt to laboratory conditions, this species is currently the subject of intensive experimental investigation.

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Materials and Methods

The zooplankton organisms used in the present program included the copepods: *Calanus finmarchicus*, *C. hyperboreus*, *Paraeuchaeta norvegica*, *Pleuromamma robusta*, *Bathycalanus* sp., *Rhincalanus nasutus*, *Euchirella rostrata;* the amphipods *Phronima* sp., *Euthemisto compressa*, and *Hyperia galba*, and an unidentified euphausid probably belonging to the genus *Thysanoessa*. All these animals are of large size and easily recognizable with the naked eye, even on shipboard. For the graphic analyses of respiratory rates use was also made of some data, hitherto unpublished in the present form, for the small neritic copepod *Acartia clausi*.

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The animals were captured with a ¾-meter or 1-meter net of mesh size #00 or #000, with a glass jar of one quart capacity secured in the cod-end. Tows were generally 15 to 30 minutes at depth, but in the case of the deeper tows the time required to reach the desired depth and to recover the net again increased the total towing time appreciably. Before the net was brought aboard after fishing, care was

TABLE I

Summary of all experimental data on the respiratory rates of zooplankton

Copepods	Location of experimental tow and estimated depth	Date	No. & stage	Experimental temp. ° C.	Dry weight/animal mg.	Respiration rate:	
						μl./animal & day	μl./mg. dry wt. & day
Calanus finmarchicus	Georges Bank 41°00′ N 67°35′ W 25 m.	Aug. 18, 1958	4 V	7.5	0.188	4.4	23.0
		Aug. 18, 1958	4 V	7.5	0.188	5.4	28.8
		Aug. 18, 1958	15 V	7.5	0.232	5.5	23.3
		Aug. 18, 1958	4 ♀	7.5	0.211	9.5	44.1
		Aug. 18, 1958	15 ♀	7.5	0.214	9.0	42.0
		Aug. 18, 1958	4 V	18.5	0.188	9.0	47.3
		Aug. 18, 1958	4 V	18.5	0.188	8.9	51.9
		Aug. 18, 1958	15 V	18.5	0.199	8.7	42.0
		Aug. 18, 1958	4 ♀*	18.5	0.211	15.7	74.2
Calanus finmarchicus	Gulf of Maine 42°25′ N 69°47′ W 200 m.	Aug. 20, 1958	15 V	8	0.294	4.7	16.2
		Aug. 20, 1958	15 V	8	0.264	3.9	14.8
		Aug. 20, 1958	15 V	8	0.334	3.6	10.9
		Aug. 20, 1958	4 V	8	0.277	5.4	19.5
		Aug. 20, 1958	4 V	8	0.277	4.7	16.9
Calanus hyperboreus	Slope water 41°46′ N 65°28′ W 1000 m.	Aug. 19, 1958	7 V	<10	1.55	18.3	11.8
			5 ♀	<10	3.68	24.8	5.5
			1 ♀	<10	3.62	26.0	7.1
Paraeuchaeta norvegica	Slope water 41°46′ N 65°28′ W 1000 m.	Aug. 19, 1958	5 ♀	<10	3.88	47.6	12.2
			5 ♀	<10	3.80	51.5	13.8
			1 ♀	<10	4.59	48.7	10.5
Pleuromamma robusta	Slope water 38°28′ N 70°59′ W 450 m.	Aug. 15, 1958	3 ♀	8	0.283	19.0	66.5
			3 ♀	8	0.283	15.5	54.3
Bathycalanus sp.	Slope water 41°46′ N 65°28′ W 1000 m.	Aug. 19, 1958	1 ♀	5.1	17.9	163.4	9.1

* 3 animals died.

TABLE I—*Continued*

Copepods	Location of experimental tow and estimated depth	Date	No. & stage	Experimental temp. ° C.	Dry weight/ animal mg.	Respiration rate:	
						µl./ animal & day	µl./mg. dry wt. & day
Rhincalanus nasutus	Slope water	April 22, 1959	2 ♀	6.5	0.826	13.0	15.7
	39°48′ N		2 ♀	6.5	1.079	15.3	14.2
	71°12′ W		2 ♀	6.5	1.019	15.0	14.7
	400 m.						
Euchirella rostrata	Slope water	April 22, 1959	3 ♀	6.5	0.890	22.4	25.2
	39°48′ N		3 ♀	6.5	0.947	28.6	34.9
	71°12′ W		3 ♀	6.5	0.820	25.6	27.0
	400 m.						
Acartia clausi	Long Island	July 22, 1953	119 ♀	5.0	0.0041	0.30	73.1
	Sound	Jan. 6, 1954	47 ♀	5.0	0.0047	0.37	78.7
	5–10 m.	Jan. 26, 1954	156 ♀	5.6	0.0073	0.67	91.8
		Feb. 2, 1954	49 ♀	5.0	0.0072	0.69	95.8
		July 3, 1954	100 ♀	5.9	0.0044	0.40	90.9
		July 10, 1954	52 ♀	5.9	0.0049	0.22	42.6
Euphausids (unidentified)	Slope water	Aug. 19, 1958	3 **	5.1	2.67	43.1	16.1
	41°46′ N		1	5.1	3.39	105.7	31.0
	65°28′ W						
	1000 m.						
Amphipods							
Phronima sp.	Slope water	Aug. 15, 1958	1	8	27.3	284.9	10.4
	38°28′ N						
	70°59′ W						
	450 m.						
Euthemisto compressa	Gulf of Maine	Aug. 20, 1958	4	4	4.97	92.7	18.6
	42°25′ N		4	4	4.87	87.5	18.0
	69°47′ W		4	4	4.35	72.3	16.6
	200 m.						
Hyperia galba	Gulf of Maine	Dec. 4, 1959	1	5.6	3.52	51.2	14.5
	42°35′ N		1	5.6	8.24	81.0	9.8
	69°35′ W		1	5.6	4.62	70.7	15.3
	250 m.		1	5.6	10.15	114.2	11.2

** 1 animal in poor condition.

taken to have on hand fresh sea water pre-cooled to a temperature at or near that of the depth fished. The cod-end was immersed in this water and the contents of the glass jar only were retained. It was assumed that animals crushed against the meshes of the net were likely to be damaged, so that the net was never washed down and the entire process of getting the organisms from their natural temperature conditions to conditions simulating them was carried out as rapidly as possible. If the temperature difference between the surface water and the depth fished was great, the mortality among the deep-water forms was high despite the precautions taken. However, the animals were sorted immediately and only the healthy specimens kept for experimental work.

On shipboard, a portable refrigerator which opened from the top was used as an experimental laboratory. In the earlier studies, some difficulty was encountered in regulating the temperature of this box when it was opened frequently. A larger expansion valve, enabling higher refrigerant pressures, eliminated this difficulty, and in its present form the box can be kept open for long periods even in summer without excessive temperature change. On return to land facilities, a constant temperature room and conventional refrigerated water baths provided supplementary, controlled-temperature conditions when required.

Since most of the animals used in this study came from water deeper than 200 meters, 5–6° C. was chosen as the temperature at which the experimental and observational studies would be run, although for reasons mentioned above this was not attained in every case (see Table I). Initially it was hoped to compare the respiration of some of the animals at 5° and at surface temperature, to enable the computation of a rough Q_{10}. However, prolonged exposure to warmer surface waters proved lethal to most of the experimental material so that except for the relatively eurythermal *Calanus finmarchicus*, such a comparative study was not possible.

Respiration was measured using glass-stoppered bottles of appropriate size. Animals were placed in a small quantity of water in a bottle, and then the bottle and contents were flushed several times with water of known oxygen content, using a siphon arrangement with a bolting cloth screen to prevent loss of the experimental material. Control bottles were prepared in precisely the same manner except that the animals were omitted. All bottles were filled completely, taking care to insure that no air bubbles were included. As Marshall *et al.* (1935) demonstrated an increase in respiration for *Calanus finmarchicus* on exposure to light, all bottles were placed in black cloth bags during the run, regardless of the experimental conditions. At the end of a suitable period of time (8 to 48 hours), single samples from the experimental and the control bottles were siphoned into smaller glass-stoppered bottles and the oxygen content determined by the Winkler method. The oxygen utilization was determined from the difference between the bottles containing animals and those without. (For a detailed description of the method, with a discussion of its advantages and disadvantages, see Conover, 1956, 1959.)

The animals themselves were generally dried for weighing while in fresh condition, or in some instances a sample of the same species and stage of animal was dried as representative of the experimental animals. Animals were weighed on a suitable quartz helix microbalance made by the Microchemical Specialties Company, Berkeley, California. In the case of the smaller organisms such as *Calanus finmarchicus* a balance with working sensitivity of 2 mg. ± 1 μg. was used. For larger forms a 20 mg. ± 10 μg. balance was employed. Aside from the accuracy of weighing in this manner, the process is extremely rapid and largely free from errors due to sudden temperature change, varying humidity, etc. In a few instances, the animals were too large for either helix and they were weighed on a conventional analytical balance.

In the respiration experiments, as well as in laboratory culture studies, antibiotics were used to control bacterial growth and respiration. Dihydrostreptomycin sulfate in concentration 50 mg./L. was generally used in respiratory studies, sometimes supplemented with 10 mg./L. of chloromycetin. Chloromycetin was found to have an inhibitory effect on the feeding of *Calanus finmarchicus* when used at 50 mg./L. (Conover, Marshall and Orr, 1959). Since oxygen utilization attributable to bacteria was generally less than 0.1 ml./L., even in 48-hour experiments, with streptomycin alone, chloromycetin was eventually eliminated altogether from the experimental procedure.

For laboratory culture experiments, the organisms were kept in polyethylene "freezer containers" of pint, pint and a half, quart, or gallon size, depending on the size and number of animals to be cultured. These containers proved non-toxic to all animals tested, and, when fitted with their plastic tops, were safe from spillage or breakage in a rough sea. For most organisms, one or two animals in the pint-sized container proved most satisfactory; the containers then could be conveniently stacked four or five high without danger of upsetting, and in this size the sides are low enough to permit easy observation with a dissecting microscope.

Sea water for cultures was passed through a type AA Millipore filter (pore size 0.80 μ), cooled, and aerated before use. In the earlier studies, the culture water was generally taken from the same area that produced the animals, but it was found that local water from Vineyard Sound was also satisfactory. Streptomycin and penicillin "G" potassium 50 mg./L. were used together at first, but later alternated at each change of culture medium to lessen the possibility of "antibiotic resistance" developing among the contaminating bacteria.

The phytoplankton organisms tried as food for the animals included *Skeletonema costatum, Thalassiosira decipiens, T. fluviatilis, Chaetoceros affinis, Rhizosolenia setigera,* and *Coscinodiscus asteromphalus,* all from laboratory cultures. Living *Artemia* nauplii and *Pinnotheres* zoeae, fresh-caught harbor copepods including *Acartia clausi, A. tonsa, Temora longicornis, Centropages hamatus, Eurytemora hirundoides,* and *Labidocera aestiva,* as well as various invertebrate larvae in the plankton were given as animal food. Bits of mussel, clam, and living and dead offshore zooplankton were also given to some of the larger carnivorous forms. The number of fecal pellets produced was used as a criterion for the amount of feeding although in a few instances change in the number of food organisms was also determined.

No attempt was made to determine the food in nature by examination of gut contents. Food passes through the animal's gut very rapidly and particularly in the case of offshore forms the gut is usually empty.

Observations on Feeding and Behavior of Animals in the Laboratory

Copepods

Calanus finmarchicus. Marshall and Orr (1955a, 1955b) have summarized what is known concerning the feeding and behavior of this species, and little can be added by this investigation. *Calanus finmarchicus* is known to eat a wide variety of diatoms, dinoflagellates, and other flagellated forms. Nannoplankton is eaten but the animal showed a decided preference for larger food (Marshall and Orr, 1955b). In addition, radiolarians, tintinnids, and crustacean remains have been found in the gut (Marshall and Orr, 1955a). In the present study, *C. finmarchicus* ate *Skeletonema, Rhizosolenia,* and both species of *Thalassiosira.* Although it may take in animal food inadvertently, it would seem that this species is primarily a herbivore.

Calanus hyperboreus. This species is generally regarded as an arctic form, where it commonly occurs in the surface waters. It is moderately abundant in the Gulf of Maine and has been observed in the slope water at depths greater than 400 meters as far south as about 38° N. Juveniles were captured a mile south of Gay Head on Martha's Vineyard, Massachusetts, in March and April, 1958.

When first returned to the laboratory, *C. hyperboreus* generally did not eat any plant or animal food readily, but after one to two weeks healthy specimens ate all phytoplankton species presented to them, including *Skeletonema costatum, Thalassiosira decipiens, T. fluviatilis, Chaetoceros affinis* and *Coscinodiscus asteromphalus.* Many large fecal pellets were produced, often two or three millimeters long, which on microscopic examination contained some green material and abundant smashed tests of the species of diatom fed.

This species is anatomically very similar to *C. finmarchicus* and is almost certainly herbivorous in northern seas. However, it is difficult to understand how it can obtain plant food in sufficient abundance to sustain it in the slope waters south of Woods Hole, Massachusetts, in summer when the waters of the euphotic zone are too warm for it. Possibly the animal goes into a state of "quiescence" when food is scarce, which may explain why no food is taken when it is first brought into the laboratory. Recent unpublished experiments suggest that the increase in activity after some days in the laboratory is also accompanied by an increase in respiratory rate.

Sømme (1934) observed that breeding animals frequently ate their own eggs. This observation has been confirmed in the present study, but it was found that egg-eating was much greater when animals had no other food available than when abundant phytoplankton was present.

Paraeuchaeta norvegica. Lowndes (1935) examined living and preserved specimens of this large copepod and concluded that it was entirely carnivorous. The animal refused all plant food presented to it in the current work. On the other hand, some of the laboratory specimens fed on small neritic copepods readily as long as they were alive. *Acartia tonsa* and *Centropages hamatus* were taken frequently, although it is questionable whether *Paraeuchaeta* would encounter either in nature commonly. It did not eat *Artemia,* though some decapod larvae were consumed. During feeding, fecal pellets were produced which contained obvious animal fragments. If both plant and animal material were fed simultaneously, the fecal pellets produced contained only animal remains.

The maxillipeds in this animal are large, prehensile and carried far forward in "praying mantis" fashion. The actual capture of the prey was not observed but the animal would seize the end of a needle or micropipette when irritated in a manner which must closely duplicate the process of food getting. The strength of the animal was surprising and a smaller copepod would have little chance of escape once it was grasped by the maxillipeds. Curiously, some of the laboratory specimens hooked their maxillipeds over their first antennae. In this position they seemed quite helpless and unable to get free. When the maxillipeds were released by the investigator, the animal seemed quite healthy, but usually caught the maxillipeds again in a few hours.

Bathycalanus sp. There can be little doubt that these large red copepods from deep water must be carnivorous, although no food, plant or animal, was taken during the three weeks they were kept in the laboratory. The female whose respiration was measured was over 13 mm. long with antennae reaching over 2.5 cm. from tip to tip. In swimming, the movements were generally unhurried, almost deliberate, but when disturbed the animal dashed about the culture vessel in a frantic effort to escape, frequently sustained for some seconds.

Rhincalanus nasutus. This species survived well under laboratory conditions and ate any species of phytoplankton offered (*Skeletonema costatum, Thalassiosira decipiens, Rhizosolenia setigera*). One female lived for three months before being accidentally killed, during which time she matured and laid numerous fertile eggs. Attempts to raise the nauplii were unsuccessful.

Euchirella rostrata. These robust-appearing copepods did not survive particularly well in the laboratory although a few fecal pellets were produced when the animals were fed on *Skeletonema, Thalassiosira decipiens* and *Rhizosolenia.* All were dead within five weeks of capture, but during this period several females matured and laid. The eggs, which were a deep purple, were remarkable for their size relative to the animal which produced them. The lengths of the cephalothorax for the captive animals ranged from about 2.9 to 3.1 mm., while the eggs produced measured over 0.4 mm. in diameter. In contrast, *Rhincalanus nasutus,* size range 4.2–4.7 mm., laid eggs about 230 μ in diameter, and in the still larger *Calanus hyperboreus,* cephalothorax 5.5–6.0 mm., the eggs ranged from 190–210 μ. *Euchirella* laid only a few eggs at a time and they were very buoyant. Unfortunately none of the eggs developed.

The density of this animal in the adult stage was remarkably high in contrast to its eggs. The animals did not swim continuously in the laboratory containers and when swimming ceased, they sank rapidly to the bottom. The carapace seemed unusually sclerosed for so small an animal, being hard and smooth to the point of a dissecting needle. Their movements were exceedingly rapid and they leaped about vigorously when out of water. One individual traveled a measured distance of 20 cm. in a single leap from the shallow dish, containing water several millimeters deep, in which it was being examined. Although it was not demonstrated that *Euchirella rostrata* does prefer animal food, the generally poor feeding on phytoplankton, robust anatomy and prehensile head appendages make it virtually certain that the animal is largely carnivorous.

Other copepods. Only a few specimens of *Pleuromamma robusta* were taken in near-surface tows over the continental slope and no observations on the organisms in captivity were made. The structure of the mouth parts and general morphology would suggest that it is largely herbivorous (George Grice, personal communication).

No attempts were made to culture *Acartia clausi* at this time but earlier studies (Conover, 1956) leave little doubt that it is primarily a herbivore. However, two observations of considerable interest were made on the closely related species *A. tonsa.*

On one occasion, some *A. tonsa* taken from Woods Hole harbor were given as food to a larger carnivorous copepod (*Paraeuchaeta norvegica*) in the company of some *Artemia* nauplii. During the experiment, an *Acartia* male was found which had firmly grasped with its head appendages an *Artemia* nauplius. When both animals were transferred to another dish for observation the male released the nauplius which was seen to have its abdomen almost totally eaten. The *Acartia* was given additional *Artemia* nauplii but died shortly thereafter without any further predation.

On another occasion, *A. tonsa* was observed in the act of feeding on a culture of large *Thalassiosira decipiens* (cells 70–85 μ in diameter). A female (cephalothorax length 0.87 mm.), lying ventral side up on the bottom of the dish, was seen to grasp single *Thalassiosira* cells with rapid movements of the maxillipeds, bringing them to the mouth region. Several times the individual cell could be seen poised on the edge of the labrum for an instant before it passed inside the animal without being broken or apparently damaged in any way. Once inside, the cells could be seen through the transparent carapace like beads on a string lined up along the foregut. The cells were carried posteriorly by a series of peristaltic movements during which they continued to be discrete, undamaged cells until quite suddenly they lost their distinct outline and seemed to fuse into a mass which soon became noticeably darker in color. The material was passed posteriorly and eventually extruded as a fecal pellet. The entire process took about 30–45 minutes, depending on which cell was timed. On examination the pellet was seen to contain only shattered frustules of *T. decipiens* and some unidentified organic matter.

Amphipods

Only two *Phronima* were taken during the program and one was dried after the respiration experiment. Despite their transparent, somewhat delicate appearance, the organisms were quite dense with a tough, sclerosed exoskeleton. The specimens studied here were found free-living in the plankton but the animal is frequently found "living" in an empty test of a salp. Most probably the organism eats the salp in whose test it is found, for it would seem poorly equipped for filter feeding.

Euthemisto compressa was found to produce an occasional fecal pellet when given phytoplankton (*Skeletonema*) but was obviously much more successful with animal food. For instance, between October 3 and October 6, 1958, a single female was observed to eat four harbor copepods and two zoeae, one nearly as large as itself. Gravid females were taken in the plankton on several occasions, and even the newly hatched young seemed carnivorous, swarming all over a piece of dead euphausid given them. *Hyperia galba* likewise is largely carnivorous and was observed to eat bits of mussel (*Mytilus edulis*), smashed snail (*Littorina littorea*) as well as living and dead copepods. This species is frequently associated with *Aurelia aurita* or other large medusae and may share the food captured by its larger host, but it can be a free-living member of the plankton community as well (Bigelow, 1925).

Both *Euthemisto* and *Hyperia* are quite dense, heavily sclerosed, and strong swimmers. Curiously, their carapaces are strongly hydrophobic and despite their density and obvious strength, they are very prone to become caught in the surface film.

Euphausids

Of all the oceanic organisms tried, the euphausids seemed to be the most difficult to keep in the laboratory. Specimens taken on August 19, 1958, were all dead by September 4, before anything could be ascertained about their food habits. On other occasions, *Thysanoessa*, *Meganyctiphanes*, and *Nematoscelis* have been observed to eat phytoplankton (*Skeletonema costatum*, *Thalassiosira fluviatilis*), but the rate of consumption would seem to be much too low to meet food requirements. These animals did not survive appreciably better than the first group.

Several workers have noted that euphausids consume a variety of foods. *Nyctiphanes couchii*, a neritic species, eats diatoms and organic detritus predominantly but also catches *Sagitta*, smaller crustaceans, and is cannibalistic in the laboratory (Lebour, 1925). Similarly, *Meganyctiphanes norvegica* consumed plant detritus when it was present in the water, but also fed on *Calanus finmarchicus*, *Paraeuchaeta norvegica* and smaller copepods (MacDonald, 1927). Very probably most euphausids are omnivorous, but it is difficult to explain their extreme sensitivity to deficiencies of the laboratory environment.

RESPIRATION IN RELATION TO SIZE OF PLANKTONIC ORGANISM

It is generally believed that the respiratory rate of poikilothermal animals is related to some power of body weight by the expression

$$R = kW^x \tag{1}$$

where R is the volume of oxygen consumed, W the body weight, k a constant for a given set of conditions, and x is the exponent, generally between 0.66 and 1.00. When oxygen consumption is plotted against weight on log log paper the data should give a straight line with a regression coefficient equivalent to x in equation (1).

Raymont and Gauld (1951) obtained a regression coefficient of 2.19 (or 2.30 with a single aberrant value removed) when log respiration was plotted against log length of the cephalothorax for four species of marine copepods ranging in length over a size range of nearly an order of magnitude. If it is assumed that weight varies as the cube of the length, then the coefficient obtained by Raymont and Gauld becomes 0.73 (or 0.77) in the form of equation (1).

In the case of nine species of small neritic copepods, Conover (1959) computed an overall regression coefficient of 0.86 for log respiration against log dry weight. For these copepods with a total size range considerably less than an order of magnitude, weight was found to vary as the power 3.17 of cephalothorax length.

The range of variation in weight of the organisms included in the current investigation is nearly four orders of magnitude and the variation in respiratory rate per organism is likewise considerable (Table I). A log log plot of respiration against weight would be expected to give a straight line relationship with a positive regression coefficient. However, if the respiration rate is first divided by the weight of the animal, the log log plot of this value R' against weight W should yield a negative regression coefficient. Of the two methods, the second shows more clearly the decrease in metabolic rate with increasing size, and its use should save a step in the calculation of energy flow and production rates.

The least squares regression on double logarithmic coordinates of the respiratory rates as μl. oxygen/mg. dry weight and day plotted against dry weight in μg., excluding only the values for *Calanus finmarchicus* at 18.5° C. and those for *Pleuromamma robusta,* gives the equation

$$\log R' = -0.35 \log W + 2.2888. \tag{2}$$

In exponential form equation (2) becomes

$$R' = 194\, W^{-0.35}. \tag{3}$$

For comparative purposes, equation (3) may be converted to the form of equation (1) by replacing R' with its equivalent R/W,

$$R = 194\, W^{0.65}. \tag{4}$$

It can be readily seen that the exponential constant in (4) is decidedly lower than that observed by Conover (1959), and also is lower than the probable exponent computed from Raymont and Gauld (1951). Weymouth *et al.* (1944) obtained a coefficient of 0.798 for *Pugettia producta* and Vinberg (1950) recorded 0.81 for

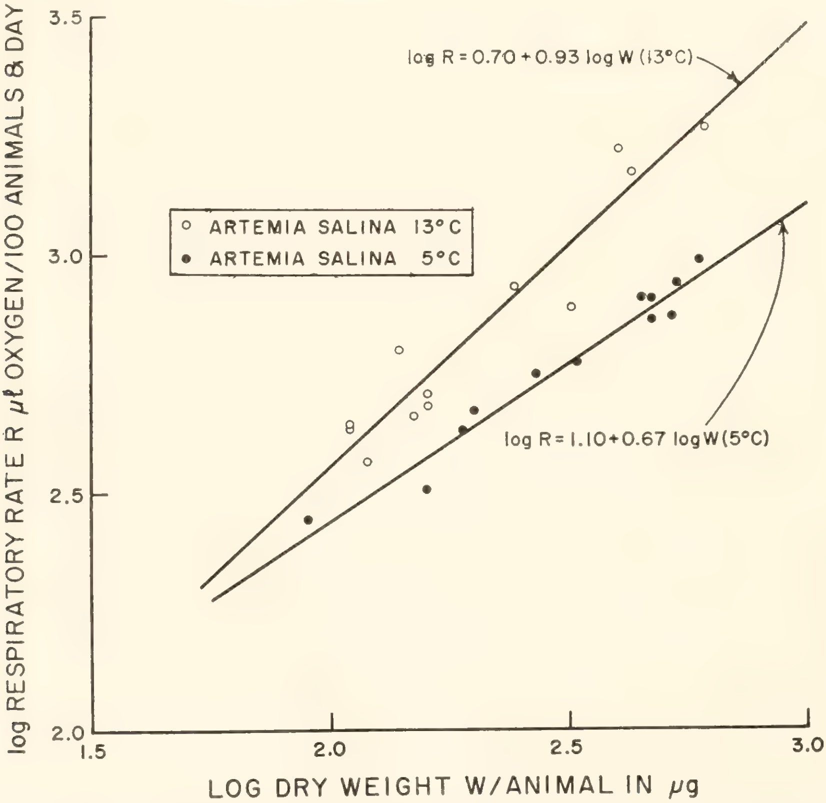

FIGURE 1. Regression lines for *Artemia salina,* showing log respiration in μl. O_2 animal and day plotted against log dry weight at 5° and 13° C.

TABLE II

Analysis of variance for regression of log respiration against log dry weight at two temperatures for Artemia salina

Null hypothesis: That there is no difference in regression coefficients for log respiration against log weight measured at 5° and 13° C.

Sources of variation		Degrees of freedom	Sums of squares	Mean square	Variance ratio
5° C.	Linear relation	1	0.319323	0.319323	173.73
	Error about line	10	0.018383	0.001838	—
13° C.	Linear relation	1	0.625688	0.625688	103.16
	Error about line	10	0.060651	0.006065	—
	Combined slope	1	0.921432	0.921432	233.17
	Between regression coefficients	1	0.023579	0.023579	5.97
	Combined error	20	0.079034	0.003952	

Variance ratio F for difference between regression coefficients = 5.97; $F_{0.975}$ (1, 20) = 5.87. Since F (1, 20) >5.87, reject null hypothesis.

Gammarus lacustris. Assuming that surface area is proportional to the square of length and that weight is proportional to the cube of length, von Bertalanffy (1951) suggested that an exponential constant of 0.667 indicates direct proportionality of metabolism to the relative surface area of the organisms. The exponential constant from equation (4) might, therefore, suggest that the surface rule applied in the case of the zooplankton investigated in the current study but not for the earlier work. However, there is also the possibility that the lower coefficient of proportionality observed here may result from the different temperature at which the experiments were run. In contrast with the temperatures of 4–8° C. used in the present work, Raymont and Gauld (1951) performed their experiments at about 17° C. and Conover (1959) used 20° as the experimental temperature. Vinberg (1950) and Weymouth *et al.* (1944) also used temperatures appreciably higher than those in the present work.

To test the hypothesis that the temperature at which a series of experiments is performed with different sized organisms might affect the proportionality of respiratory rate to size, an initial experiment with three species of calanoid copepods, ranging in weight from 0.017 to 5.45 mg., was set up at two temperatures, 5° and 13° C.; however, the scatter around the least squares regression lines was too great to permit disproof of the null hypothesis that there was no difference in the log log regression coefficient of respiration against weight at the chosen temperatures. A second experiment at the same two temperatures was then performed with a single species, *Artemia salina*, which can be cultured in the laboratory so as to supply a number of different size classes. The least square regression lines for log respiration against log weight for *Artemia* at 5 and 13° C. are shown in Figure 1. The regression coefficients, 0.67 at 5° C. and 0.93 at 13° C., can be demonstrated to be statistically different at $P = 0.025$ (Table II). According to von Bertalanffy (1951) the two regression coefficients obtained here for the same animal would be indicative of two very different metabolic types.

So long as the Q_{10} for any temperature change is the same for an animal over its entire size range, the regression of respiration against weight should give the same coefficient of proportionality regardless of the experimental temperature. In this regard, Rao and Bullock (1954) reviewed data from several sources, and concluded that the Q_{10} of various measures of activity commonly increases with increasing size over the range of ordinary physiological temperatures, although there were several cases in which the trend was reversed. *Artemia* was not one of the animals considered by Rao and Bullock, but the present data would seem to suggest that this animal does have a Q_{10} which varies with size.

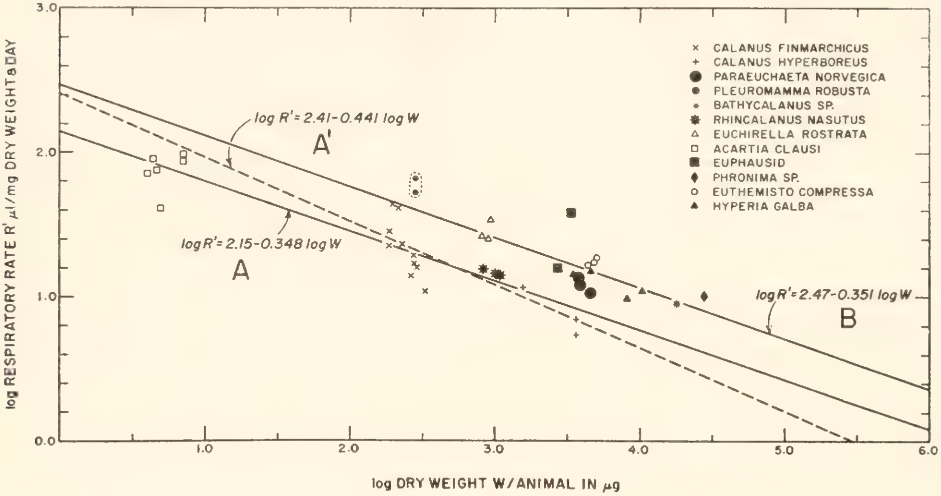

FIGURE 2. Scatter diagram and regression lines showing the relation between log respiration in µl. O_2/mg. dry weight and day and log dry weight in µg. Line A is fitted to data for suspected herbivores. Line A′ is fitted to data for herbivores, omitting values for *Acartia clausi*. Line B is fitted to data for suspected carnivores. See text for further explanation.

In the case of studies involving several different organisms taken from nature, there is the additional complexity of species differences in Q_{10}. Rao and Bullock (1954) also showed that the habitat temperatures of the animal prior to examination could affect Q_{10}. In this regard, Berg and Ockelmann (1959) observed a seasonal shift in the size-respiration relationship for the fresh-water snail *Lymnaea palustris*. Other factors, such as nutritional status, reproductive activity or some endogenous rhythm, might lead to increased variability in the observations. The resultant of one or several factors of the sort described here might be to increase or decrease the spread of values at one end of the temperature scale while having the opposite effect at the other end, regardless of the size of the animals being studied. Both the slope of the regression line and the scatter of points around it would be affected.

RESPIRATION IN RELATION TO FOOD HABITS

It was noted early in the study that *Calanus hyperboreus* and *Paraeuchaeta norvegica* taken in the same tow and studied under the same conditions had different respiratory rates despite their similarity in size and weight (see Table I). Both animals were kept in the laboratory for a lengthy period, and it became obvious that one, *Calanus hyperboreus*, was principally herbivorous while the other was entirely carnivorous. Raymont (1959) found that *Tortanus discaudatus,* also believed to be a carnivore, appeared to have a higher metabolic rate in relation to its body size than other copepods inhabiting the same type of environment.

In Figure 2 the respiratory rate at low temperatures for all the animals studied has been plotted against their weight on double log paper. On examination of the scatter diagram it seemed that the suspected carnivores in general had higher rates than the known herbivores. To test this hypothesis, separate regression lines were fitted to the data for the herbivores, *Calanus finmarchicus, C. hyperboreus, Rhincalanus nasutus*, and *Acartia clausi* (line A), and a second line fitted for the suspected carnivores, *Paraeuchaeta norvegica, Bathycalanus, Euchirella rostrata, Phronima, Euthemisto compressa, Hyperia galba*, and the euphausids (B). For the herbivores an additional regression line was also computed, omitting the data for *Acartia* since it had been acquired originally for different reasons (A′). Obviously the slope of the regression lines for the herbivores (including *Acartia* data) and the non-herbivores is not significantly different (0.348 and 0.350, respectively). With the *Acartia* data omitted the slope of the regression line fitted to the herbivore data becomes 0.441, but it can be shown statistically (Student's t test) that the slopes are still not significantly different.

TABLE III

Analysis of covariance for respiratory rates of herbivores (Acartia clausi *included*) *and non-herbivores*

Null hypothesis: That there is no difference in the respiration rate per mg. of dry body weight for herbivorous and non-herbivorous zooplankton

Sources of variation	Degrees of freedom	Sums of squares for respiration	Sums of products for respiration and weight	Sums of squares for weights	Errors of Estimate		
					Sums of squares	Degrees of freedom	Mean square
Total	38	3.76580	−10.74861	43.19223	1.09095	37	
Between animals having different food habits	1	0.38487	−2.80085	20.38336			
Within each type	37	0.38093	−7.94776	22.80887	0.61153	36	0.01699
For test of significance of mean respiration with effect of weight removed					0.47942	1	0.47942

Variance ratio $F = \frac{0.47942}{0.01699} = 28.2$; $F_{0.999}(1, 40) = 12.61$. Since $F(1, 36) > 12.61$, reject null hypothesis.

To test the null hypothesis that there is no difference between the regression lines for herbivores and non-herbivores, an analysis of covariance was employed as shown in Table III. Since the slope for the herbivorous animals was not affected by removing the *Acartia* data, it was decided to include this in the overall analysis. It is one of the advantages of covariance analysis that differences between groups of measurements can be tested in a single operation without the effect of other measurements that would normally complicate the interpretation. Thus, errors of estimate are calculated from the sums of squares and sums of products in such a way that the variance ratio tests only the respiratory rates after adjustment to remove the effect of the variable weight. Since the F test suggests that the distribution of respiration rates in relation to feeding type observed here would occur by chance much less than one time in a thousand, clearly the reason for the distribution bears some consideration.

Certain of the non-herbivorous zooplankton organisms studied were observed to be quite dense, with a hardened exoskeleton. Thus, organisms such as *Euchirella rostrata*, *Euthemisto compressa*, and *Hyperia galba* are presumably carrying around a considerable weight of inert organic material. More muscle protein, also quite dense, and more energy would be required to maintain the organism in the water column against its negative buoyancy.

There is little doubt that many herbivorous copepods also carry a high portion of their body weight as inert organic material, but in this case the substance may be oil with a positive buoyancy. In the case of the large *Calanus hyperboreus*, copepods of approximately the same external dimensions may differ in dry weight by several hundred per cent because of differences in the amount of stored oil.

It is also probable that a carnivorous animal is normally more active than an herbivorous one, regardless of their basal metabolic rates. The predator has to move about actively in search of the prey and then must overcome the natural reluctance of the prey to be caught by using its greater physical strength and swiftness. On the other hand, the prey organism in this association is more often than not the herbivorous copepod which can feed while it swims with a more or less continuous expenditure of a smaller amount of energy, since its food has at best extremely feeble power of escape.

Finally the possibility remains that a real difference could exist in the form of the organic matter oxidized by the herbivore and non-herbivore. Thus, an organism which burns carbohydrate exclusively would use decidedly less oxygen per unit of carbon oxidized than one which metabolizes oxygen-poor fats.

Before leaving the subject of respiration, a few remarks should be made concerning *Pleuromamma robusta* and its somewhat enigmatic position metabolically. As remarked earlier, it would appear that this species belongs in the herbivorous group, but so far as its respiration is concerned the organism would seem to be more closely allied to the non-herbivores. Clearly, with only two points on the graph it is not certain that any real difference exists between this and other herbivorous forms so far as respiration is concerned, and yet it remains possible that the very high metabolic rate observed for this form may be in some way related to the fact that it has a powerful bioluminescent organ. The species should certainly be given further attention.

Discussion

There is a persistent tendency for the biologist working with the most complex of organized systems to seek a simple solution or approximation which holds for the majority of cases. Thus certain scientific "laws" as $Q_{10} = 2$, metabolism equals a constant times weight to some power between 0.7 and 0.8, weight equals cube of length, and so on have come into common usage in environmental studies. Most of these "laws" had their origin in laboratory studies on mammals and they need re-examination before being applied to many ecological situations. It would be a great advantage to the biological oceanographer to have a single expression which would predict the metabolism of a population of organisms with a given size distribution under any temperature conditions, but as more data become available the evidence would suggest that such a prediction equation, if it is ever formulated, will give a high-speed computer a good workout.

The relationship between metabolism and size in the warm-blooded organisms on the average has the form

$$M = 70W^{0.74}\ \text{cal./day}, \tag{5}$$

but as Kleiber (1947) admits, the basis for this relationship is not really understood for the best known group of mammals. In the case of warm-blooded organisms metabolism is usually measured at or near the same temperature and the necessity to maintain this temperature against external environmental temperatures often very different would seem to give some importance to the relative surface area across which heat would be lost or gained.

In the sea, among the cold-blooded organisms there is tremendous variety in body form, biochemical mechanism and chemical composition. The surface of the marine organism is important for several reasons. Besides heat, nutrient substances, excretory substances, and gases pass back and forth across body membranes, and the frictional resistance of the surface area with the surrounding medium is critical for all organisms which move from place to place, whether they swim or are carried by their environment. It would seem very coincidental indeed should all the myriad forms of life be governed by a law derived from the warm-blooded organisms.

Among the marine invertebrates these problems have been examined in detail only for the Crustacea, and here it is true that there are relationships between metabolism and size very similar to those derived for homeothermal organisms. However, the effect of environmental temperatures on these relationships has not been given much prior consideration.

That other factors besides size and the direct effect of temperature influence respiratory rates of zooplankton forms has also been demonstrated recently. Conover (1956) observed a possible seasonal adaptation to the changing temperature regime for *Acartia clausi* and *A. tonsa.* Subsequently Conover (1959) and Marshall and Orr (1958) demonstrated seasonal differences in the respiration rate of several neritic species. Conover (1959) also demonstrated a significant difference between the respiratory rate of *Acartia clausi* in Long Island Sound and in Southampton Water. It can be seen from Table I of this study that *Calanus finmarchicus* had a decidedly higher respiratory rate on Georges Bank than in the Gulf of Maine. In this case, the populations are separated by not more than a hundred miles of water. To this list can now be added the difference in metabolism between organisms belonging to the same community but differing in their position within the food chain.

As a final note of complexity to the already confused picture of metabolic relationships for the planktonic organisms, it must be emphasized that even though reliable data were available for respiration per unit mass for a group of organisms, conversion of this information to food requirements or energy flow might introduce an appreciable error, due to insufficient knowledge regarding the nature of the food substance oxidized. It is well known that an animal requires more oxygen to oxidize fat than to oxidize carbohydrate. Although more energy is produced per volume of oxygen used in the case of carbohydrate metabolism, there is also more energy available per gram of fat than per gram of carbohydrate. Raymont and Conover (unpublished data) observed that several zooplankton organisms oxidized carbon in some form at a rate of at least ten times greater than changes in their carbohydrate reserves would predict. For instance, in the case of *Calanus hyperboreus*, the respiration rate was equivalent to an oxidation of 10 to 20 μg. of carbohydrate per day, an amount somewhat greater than the total carbohydrate content of the animals, and yet there was no detectable change in the total sugars.

It is becoming increasingly popular among environmental biologists to think of production as a single dynamic process which can be made to conform to some idealized mathematical model. Such an approach has had useful application, for instance in the North Sea pelagic fishery studies by Cushing (1955, 1959). Yet, as Steele (1960) points out, herring eat *Calanus* but they do not eat salps, even though each has a similar position in the food chain. Perhaps it is fortunate that salps are not particularly abundant in the North Sea! In any event, there would seem to be a good argument in favor of an increased emphasis on certain qualitative aspects of energy dynamics and food relations in the marine environment as a supplement to the purely quantitative approach.

SUMMARY

1. The respiration rate of twelve species of zooplankton, the majority from oceanic waters and from depths greater than 100 meters, has been measured at temperatures close to that of their environment (4–8° C.). In most cases healthy specimens were brought to the laboratory and their food habits and behavior studied.

2. The following species seemed largely herbivorous: *Calanus finmarchicus*, *C. hyperboreus*, and *Rhincalanus nasutus*.

3. The copepod *Paraeuchaeta norvegica*, and the amphipods *Euthemisto compressa* and *Hyperia galba* all took animal food readily. *Bathycalanus* sp., *Euchirella rostrata* and the euphausids also are believed to be at least partially carnivorous although they demonstrated little or no feeding.

4. The animals studied had a total range in dry weight of nearly four orders of magnitude. When log respiration was correlated with log weight, a positive linear regression coefficient of 0.65 was obtained. This value, which is lower than most previously determined regression coefficients relating size and metabolism in the Crustacea, may result from the lower temperatures used in these experiments compared with those used in the earlier work.

5. As confirmatory evidence, size and metabolism were related by a coefficient of 0.67 at 5° and 0.93 at 13° in the case of *Artemia salina*.

6. Those zooplankton animals which seemed to be largely carnivorous on the basis of the behavioral studies had a significantly higher respiratory rate than those which seemed to be predominantly herbivorous.

7. Some of the possible explanations and ecological implications of the abovementioned observations are discussed.

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THE PHYSIOLOGY OF SKELETON FORMATION IN CORALS. IV. ON ISOTOPIC EQUILIBRIUM EXCHANGES OF CALCIUM BETWEEN CORALLUM AND ENVIRONMENT IN LIVING AND DEAD REEF-BUILDING CORALS

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The experiments reported here were carried out to determine the extent to which the polyp layer covering the outside of a coral colony can act as a barrier cutting down or preventing altogether the equilibrium exchange of calcium between the corallum and the environment.

The occurrence of a calcium equilibrium exchange process between the skeletal mineral of corals and the sea water was described in a previous paper (Goreau, 1959a), but in comparing the calcium-45 uptake of living corals with that of dead macerated pieces of corallum, we observed that the incorporation of the radioactive calcium by exchange was in most cases much slower than that deposited by active secretion in living corals.

The total rate of exchange between the solid and dissolved phases in a system at equilibrium depends on the temperature and on the surface area of the solid phase in free contact with the medium. The former variable will not be considered further in this study since the temperature of most West Indian coral reefs remains stable within the range of 25° C. to 29° C. On the other hand, the total surface area that is potentially available for exchange in an average coral colony is enormous, due to the fact that the corrallum is not a solid mass of mineral matter, but a complex framework of interlocking calcareous lamellae with water-filled spaces in between. In the Scleractinia, the mineral structure is composed of more or less trabeculate spherulitic aggregates of aragonite crystals (Wells, 1956), with the living polyps located only on the external surface of the skeletal mass. In eight Indo-Pacific reef-building corals Odum and Odum (1955) estimated that the pores occupied between 7% and 38% of the total skeletal volume. Consequently, while the internal surface area of the coral skeleton must be very large it is questionable whether more than a very small fraction of this can freely exchange with the surrounding environment, since most of the outer surface of living corals is covered with a continuous sheet of polyps which tends to isolate the skeletal mass from direct contact with the sea water. Exposure of the skeleton to the medium can occur only where the polyparium has died and disappeared, but secondary encrusting organisms rapidly overgrow these parts of the colonies with a thick layer of their own.

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The water occluded in the internal pore spaces of the corallum is not in free communication with the external medium and exchanges only very slightly with it. This is demonstrated by our observations that the pore water inside of living corals immersed in sea water enriched with calcium-45 for 12 to 24 hours contains virtually no radioactivity (Goreau, unpublished). The huge internal surface area of the crystalline matrix of corals is effectively barred from exchanging with the environment by the paucity of its connections with the external medium, which means that the internal environment of a living coral head is relatively isolated from the outside.

The surface waters of the tropics are saturated with respect to aragonitic and calcitic $CaCO_3$. At the temperatures and salinities normally encountered in a coral reef, the solubility product of aragonite is quite large, about 10^{-6} (Revelle and Fairbridge, 1957). Hence, the corallum, which is composed of nearly pure aragonite (Meigen, 1903; Lowenstam, 1954; Chave, 1954), will undergo chemical exchange with the aquatic environment according to the reaction

$$CaCO_{3\,(s)} \rightleftharpoons CaCO_{3\,(l)} \rightleftharpoons Ca^{++} + CO_3^{--}, \text{etc.}$$

No net deposition or solution can occur as long as the system is in chemical equilibrium, but when the solid phase is enriched in calcium-45 and is in contact with freely flowing sea water saturated with $CaCO_3$ containing no calcium-45, then isotopic equilibrium cannot be established and the radioactive calcium will be lost from the coral at a rate directly proportional to the rate of the chemical equilibrium exchange. This condition was produced by "infecting" living corals with calcium-45 and then measuring the rate at which the isotope was lost when the living colonies were placed back on the reef and allowed to resume growth under natural conditions for a prolonged period of time.

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Loss of Calcium by Exchange from Living and Dead Corals

The observations cited in this section were carried out on colonies of *Porites furcata* (Lamarck) and *Millepora alcicornis* (Linnaeus). The latter is not a scleractinian coral but a hydrocoral belonging to the order Milleporina of the class Hydrozoa (Hyman, 1940). These organisms secrete a massive aragonitic skeleton; in the West Indies the Milleporina are important reef-builders in association with the shallow water Scleractinia (Goreau, 1959b).

For the purpose of these experiments we chose large and symmetrical colonies growing at a depth of about five feet on the windward reef at Magueyes Island, which is situated off the southwest coast of Puerto Rico. The symmetry of these colonies indicated that they were growing at about the same overall rate in all directions. Two whole specimens of each were carefully detached from the reef and transferred into large plastic tubs without being allowed to come into contact with the air. These were ferried by boat to the nearby laboratory jetty where, still in their original containers, they were put under a twin bank of 80-watt fluorescent lights located about 18 inches above the specimens. The water was stirred and aerated by a stream of saturated air. After a 24-hour period of acclimatization neutral $Ca^{45}Cl_2$ was added to a final activity of about 0.14 μc/ml. The corals were exposed to this concentration of calcium-45 for 48 hours. For the purpose of measuring specific activity, 50-ml. water samples were withdrawn one, 24 and 48 hours following addition of the isotope. The water temperature throughout this period was 28 ± 1.5° C. At the end of the run, the corals were transferred to a large wire mesh cage anchored on the reef in approximately 4½ feet of water, close to the site from which they had originally been collected and where the ecological conditions were the same. The bottom of the cage, made from one-inch galvanized iron mesh wire, was raised about 12 inches off the sea bottom by wooden legs, to protect the specimens from sediment, and at the same time expose them adequately to the prevailing water currents.

Samples were obtained by breaking off groups composed of 10 to 15 individual branches from each colony. The first samples were taken three hours after removal of the corals from the radioactive sea water. Fourteen samples of *P. furcata* were taken over a period of 23 days. *M. alcicornis* was sampled nine times over the first ten days, and twice more at 154 and 156 days.

The samples were cleaned of all worm tubes, ophiuroids, sponges, algae and other encrusting organisms, and then dried at 85° C. for one week. In the case of the *P. furcata* samples, the upper living parts of the branches covered by the dark brown polyparium were separated from the older greenish grey parts which were dead. The two series of samples were kept for separate analysis. A parallel series of dead samples was not obtained from *M. alcicornis* because the entire colonies were covered by a sheet of living polyparium.

The cleaned and dried samples were dissolved in concentrated HCl after addition of 1 ml. octyl alcohol to reduce the violent foaming. The suspension was homogenized, boiled, cooled and made up to a standard volume with double distilled water; the calcium-45 activity was determined on duplicate 1-ml. aliquots using the oxalate precipitation method described in a previous paper (Goreau, 1959a). All results were expressed in counts per minute per milligram calcium, *e.g.* the specific activity. Due to the long duration of this experiment, the activities measured were corrected for radioactive decay, using the time of removal of the corals into inactive sea water as zero time.[2]

The results of these experiments are summarized in Table I. Each datum represents the mean specific activity of a group of 10 to 15 individual branches broken off at random from the test colonies at the times indicated in the left-hand column of the table. The mean specific activity only is given for each group since the individual branches constituting each sample were pooled together.

As is shown in Figures 1A and 1B for *P. furcata*, there is considerably more scatter of our results in the samples taken from the living parts of the corals, where

[2] The half life of calcium-45 is 163 days.

most of the calcium-45 was incorporated by active deposition, than in the dead samples in which the calcium-45 content was due entirely to equilibrium exchange. Such variations, characteristic of living corals, are due to short term inequalities of the calcium deposition rates in different parts of the colonies tested, and may be evidence of a discontinuous growth process (Goreau and Goreau, 1959). Had these corals been exposed to the calcium-45 for a considerably longer period than 48 hours, the variations in the specific activity would probably have been much smaller, or even absent. From the symmetrical appearance of the test corals used, we assumed that the average growth rate was on the long run about the same in all parts of the colonies. Since these corals were exposed to calcium-45 for only two days, the pattern of random variation in the growth rates that existed during this time was essentially "frozen" when the colonies were removed into inactive sea water.

TABLE I

Changes in Ca^{45} activity with time in living and dead corals. The figures are specific activities in counts $min.^{-1}$ $mg.$ Ca^{-1}

Time in days	*Porites furcata*		*Millepora alcicornis*
	Live	Dead	
0.125	255.9	55.8	66.4
1	202.6	33.8	89.2
2	266.8	30.8	
3		35.2	54.1
4			56.3
5			45.0
7	244.4	19.1	70.1
8	274.3	25.1	50.1
9			49.9
10			52.6
12	267.2	22.7	
13	252.9	24.4	
14	265.8	18.7	
17	286.8	17.8	
18	277.3	19.5	
19	248.3	20.2	
21	234.4	14.2	
22	230.5	10.6	
23	237.2	19.5	
154			38.2
156			76.4

In Figure 1, the changes in activity with time are shown for *P. furcata:* curve A represents the living parts, curve B the dead branches. The regression lines drawn through the points were fitted by the method of least squares. The distribution of points in the case of the living branches of both *P. furcata* (Fig. 1A) and *M. alcicornis* (Fig. 1C) is such that an attempt to derive a general relationship for the rates of change of the specific activities with time yielded meaningless equations, and the slopes of the regression lines are too small to be significant on the basis of the available data. This means that there was no significant loss of calcium-45 by isotopic exchange from the living parts of both species tested over the time of our observations, which was 23 days for *P. furcata* and 156 days for *M. alcicornis*.

As seen from the curve in Figure 1B, the situation was very different in the dead parts of *P. furcata* where the naked corallum was directly exposed to the circulating sea water. There is a progressive decrease of the radioactivity with time, due to loss by isotopic exchange. Because of the smaller scatter of the data the fit of the regression curve to the points is quite good. The line yields the equation

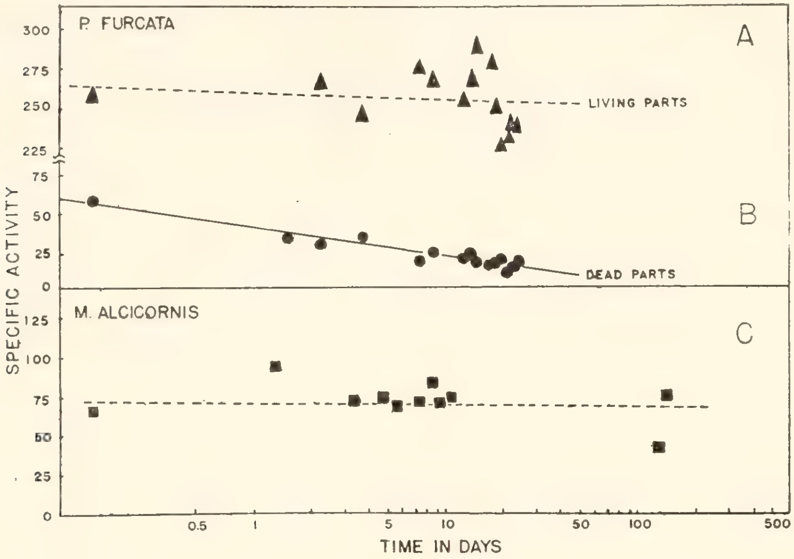

FIGURE 1. Changes of specific activity as a function of the time in the skeletons of living and dead corals, plotted on semi-logarithmic coordinates. Curves A and B represent the activity due to calcium-45 over a period of 23 days in living and dead *Porites furcata*, respectively. Curve C shows the same in *Millepora alcicornis* over a period of 156 days. The regression lines were fitted by the method of least squares. Note that no significant loss of calcium-45 activity occurs in the living corals, but in the dead parts of *Porites furcata* the slope of the curve shows that the exchangeable calcium-45 activity has a half-life of approximately five days.

$$A = 200\, e^{-0.136\,T}, \qquad (1)$$

for the relation between the specific activity of the corallum (A) and the time (T). The slope of the curve shows that one-half of the original activity on the surface of the dead parts of *P. furcata* colonies is lost in about five days, and nearly all in about 110 days.

CALCIUM EXCHANGE IN DEAD CORALS WITH AND WITHOUT COENOSARC

The effect of the mechanical presence of a tissue barrier on the calcium exchange in corals was tested by comparing isotopic equilibration rates in killed corals, in which the coenosarc was still present, with that in corals from which the fleshy parts had been removed by maceration.

Corals of several different species were killed and preserved in sea water containing 4% formaldehyde buffered to pH 8 with bicarbonate. Each specimen was split into two pieces, one being preserved in the formaldehyde while the other was macerated in sea water until the tissue layer covering the outside of the corallum was completely removed. The clean macerated halves were then placed together with the preserved halves into the buffered sea water-formaldehyde solution, and neutralized $Ca^{45}Cl_2$ was added to a final activity of approximately 0.05 μc./ml. The medium was stirred with a stream of saturated air pumped through an airstone. The corals were allowed to soak in the radioactive sea water for 48 hours, the temperature being maintained at 28 ± 1° C. during this time. Samples of water were withdrawn for measurement of the specific activity at the beginning and at the end of the runs. The pieces of coral were drained on filter paper to remove the most of the adherent radioactive water, rinsed in six changes of large volumes of inactive sea water over a period of about 15 minutes and allowed to drain for eight hours on blotting paper. All specimens were treated exactly the same way.

TABLE II

Exchange uptake of Ca^{45} by killed corals with and without coenosarc. Average specific activities in counts $min.^{-1}$ $mg.$ Ca^{-1} ± S. D. Number of colonies sampled in brackets

Species	Macerated colonies without coenosarc	Colonies with coenosarc
Acropora cervicornia	11.20 ± 1.00 (4)	10.10 ± 0.80 (4)
Manicina areolata	0.82 ± 0.10 (5)	1.78 ± 0.10 (5)
Montastrea annularis	1.70 ± 0.20 (4)	1.70 ± 0.10 (4)
Montastrea cavernosa	1.00 ± 0.20 (4)	1.10 ± 0.05 (4)
Diploria strigosa	0.60 ± 0.05 (4)	1.50 ± 0.06 (4)

Sampling was carried out by cutting off 3-cm.-long pieces from the terminal parts of branching corals, or taking core samples with a hollow punch from the massive corals (*cf.* Goreau and Goreau, 1959). To make the data from macerated and unmacerated specimens as comparable as possible, great care was taken to sample only strictly corresponding areas of the colonies. Three pieces were removed from each test specimen. These were once more rinsed in fresh sea water for 30 seconds, drained on filter paper, and dissolved in individual flasks containing concentrated HCl. The samples were homogenized, boiled, cooled, made up to a standard volume and the radioactivity determined by the oxalate method on replicate 1-ml. aliquots. Due to the low activities of the same samples, the time taken for 2000 counts was used as a basis for our measurements, and the results expressed as counts per minute per milligram calcium.

The results of these experiments are given in Table II for five species of reef-building corals. The numbers represent the specific activities; each value is the mean of three measurements. The numbers in brackets correspond to the number of separate colonies tested.

In three of the five species tested, there were no significant differences in the amount of calcium-45 taken up by the macerated specimens as compared with those still having a mechanically intact layer of tissue. The brain corals, *Diploria strigosa* and *Manicina areolata,* were the exceptions to this; significantly higher specific activities were observed in the preserved colonies which still had the intact coenosarc. As there is no evidence that coral tissues contain substances which bind inorganic calcium, we believe that the increased activity was due to occlusion in the coral of a small amount of radioactive sea water that was not washed out completely. The rinsing procedure was kept as short as possible to prevent loss of calcium-45 from the specimens by exchange with the wash water.

These data indicate that the mere mechanical presence of an intact, but dead, tissue layer does not appear to hinder significantly the isotopic exchange of calcium between the corallum and medium over a period of 48 hours.

A notable feature of these results is that much more calcium-45 was exchanged by the skeleton of *Acropora palmata* than by any of the other corals tested. This is due to the greater porosity and surface area in the corallum of the Acroporidae as compared with that of the Faviidae, the family to which all the rest of the corals listed in Table II belong. The isotope exchange method may eventually become useful as a quantitative measure of the skeletal surface area and porosity in different corals if suitable reference standards can be developed.

Calcium Exchange in Corals With Extremely Low Growth Rate

The experiments described in this section were conducted on colonies of *Oculina diffusa* in which the active calcium uptake rate was depressed to extremely low levels through the combined removal of the zooxanthellae and treatment with acetazolamide (2-acetylamino-1,3,4-thiazole-5-sulfonamide) to inhibit the enzyme carbonic anhydrase (*cf.* Goreau, 1959a). The calcium-45 deposited by these corals was compared with that taken up through isotopic exchange by control colonies of the same species which had first been killed and macerated to remove the tissue layer covering the skeleton.

Zooxanthellae were removed from the corals by keeping the colonies in darkened aquaria for two months. The corals fed on zooplankton brought in from the outside by the circulating fresh sea water supply showed no signs of starvation from this treatment. Complete extrusion of the zooxanthellae caused the coenosarc to change from a translucent yellowish brown to a transparent colorless appearance. The "bleached" corals were healthy and fully expanded at all times, but their calcium deposition rates were extremely low in comparison with normal colonies of the same species containing a full complement of zooxanthellae (Goreau, 1959a; Goreau and Goreau, 1959, 1960).

The zooxanthella-less corals and the macerated controls were distributed into four glass jars, two of which were painted black to exclude light. One light-dark pair contained only fresh sea water, the other contained sea water with 10^{-3} M acetazolamide.[3] For the 48-hour duration of the experiment, the jars were kept under a twin bank of 80-watt fluorescent tubes, the temperature was maintained at 28 ± 0.5° C. and the contents of the jars were stirred and aerated with a fine stream of saturated air.

After the corals were exposed to the action of 10^{-3} M acetazolamide for 24 hours, neutral $Ca^{45}Cl_2$ was added to a final concentration of about 0.1 μc./ml. Multiple samples of the living experimental colonies and the dead controls were taken at 3-, 7-, 12- and 15-hour intervals after addition of the isotope. Water aliquots were taken at the same times to measure the specific activity of the medium. The coral samples, taken by breaking off terminal portions of the branches, were immediately drained on filter paper, rinsed in six changes of fresh inactive sea water, drained again and dissolved in dilute HCl (1 : 1) in individual volumetric tubes. Analysis for calcium-45 activity was done by the oxalate method and the results calculated in terms of the specific activity, *e.g.* counts per minute per milligram calcium. At the end of the experiment the remaining corals which had been in the acetazolamide solution for a total of nearly 40 hours appeared as healthy as the controls kept in plain sea water. The colonies were fully expanded and able to ingest small pieces of clam meat. When returned to fresh sea water, they lived for a further two weeks until sacrificed for histological purposes.

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[3] Kindly supplied as "Diamox" by the Lederle Laboratories Division of the American Cyanamid Company.

TABLE III

Calcium-45 activities in living zooxanthella-less Oculina diffusa compared with dead control colonies, in light and darkness, and in presence or absence of acetazolamide. Figures represent the specific activities in counts min.$^{-1}$ mg. Ca^{-1} ± S.D. Number of samples in brackets

Time in hours	Plain sea water			
	Light		Dark	
	Living corals	Dead corals	Living corals	Dead corals
3	1.9 ± 0.1 (4)	2.6 ± 0.3 (3)	1.4 ± 0.2 (4)	1.6 ± 0.2 (3)
7	5.3 ± 1.0 (5)	3.5 ± 0.2 (3)	3.0 ± 0.8 (3)	2.7 ± 0.1 (3)
12	9.4 ± 0.6 (3)	3.6 ± 0.2 (3)	3.1 ± 0.3 (5)	4.0 ± 0.1 (3)
15			3.8 ± 0.5 (4)	4.2 ± 0.05 (3)
	10^{-3} M acetazolamide			
3	1.4 ± 0.1 (4)	1.6 ± 0.2 (3)	1.2 ± 0.01 (4)	3.7 ± 0.1 (3)
7	0.95 ± 0.3 (6)	3.0 ± 0.3 (3)	1.4 ± 0.1 (4)	4.4 ± 0.1 (3)
12	1.9 ± 0.6 (5)	3.3 ± 0.05 (3)	1.8 ± 0.2 (3)	5.0 ± 0.05 (3)
15	1.3 ± 0.6 (4)	3.8 ± 0.2 (3)	2.0 ± 0.5 (4)	4.9 ± 0.2 (3)

The results of these experiments are given in Table III. In the absence of acetazolamide, the zooxanthella-less corals which had been kept in darkness (Fig. 2B) contained about the same specific activity as the dead controls, whereas those growing in light had more than twice the activity of the controls (Fig. 2A). The reason for this is not clear since zooxanthellae appeared to be completely absent from these colonies. Some potentiation of the growth rate might have occurred if boring algae were still present, but no chlorophyll tests were made on these specimens at the time. In previous experiments on this and other corals from which zooxanthellae had been removed, we found no significant differences of the calcium uptake rates in light and darkness (Goreau, 1959a).

In the presence of 10^{-3} M acetazolamide, the calcification rate of the experimental colonies was so reduced that they contained less than half of the specific activity of the dead controls. The effect was the same in light as in darkness (Figs. 2C and 2D). Whereas the activity of the dead controls increased continuously for the first 12 hours, the activity of the living test corals showed little

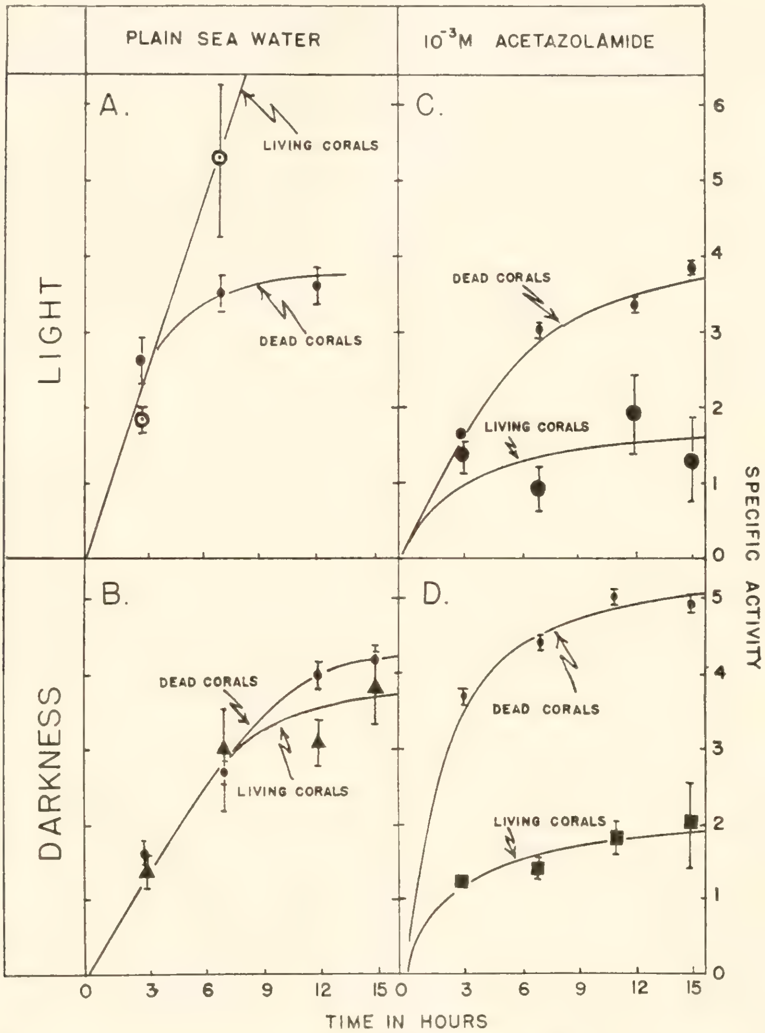

FIGURE 2. The effect of the inhibition of carbonic anhydrase by 10^{-3} M acetazolamide on the rate of calcium-45 incorporation by zooxanthella-less *Oculina diffusa*, compared with the rate of isotopic exchange in dead macerated control colonies, in light and in darkness. Calcium-45 incorporation by living and dead corals in plain sea water is shown in Figures 2A and 2B. Figures 2C and 2D show the same in the presence of 10^{-3} M acetazolamide. Note that in the presence of the carbonic anhydrase inhibitor the specific activity of the living corals is less than half that of the dead exchange controls.

increase beyond that incorporated during the initial three hours of the experiment. We consider it probable that most of this activity was due to a small amount of calcium taken up into the tissues but not deposited into the skeleton. It is clear from these results that the living zooxanthella-less colonies in which the enzyme carbonic anhydrase was also inhibited probably did not grow at all. However, due to the presence of the living coenosarc, the process of isotopic exchange between the skeleton and medium was very much slower than in the dead controls in which the skeletal surface was in direct contact with the medium and so free to exchange with it.

Discussion

The equilibrium exchange rate of calcium between the coral skeleton and the surrounding sea water is very different in dead corals as compared to living corals. We have found earlier that isotopic equilibration between corallum and sea water enriched with calcium-45 is more or less complete in about 48 hours at a temperature of 28° C. (Goreau, 1959a). However, corals in which the calcification rate was reduced to very low levels by the simultaneous removal of zooxanthellae and inhibition of carbonic anhydrase with acetazolamide contained less radioactivity than macerated "exchange controls." Our data indicate that the mechanical presence of the coenosarc does not hinder calcium equilibrium exchange if the tissues have been killed. On the other hand, the living coenosarc is comparatively calcium-proof, thus preventing the underlying skeletal mass from exchanging with the surrounding environment. This is supported by our previous observation that the living polyps of *Astrangia danae,* a cold-water coral, tend to exclude calcium relative to the sea water in which they are growing (Goreau and Bowen, 1955).

In corals growing under natural conditions on a reef the exchange rate of calcium between the skeleton and the medium is much higher in the dead parts of the colonies which are unprotected by the polyparium than in those parts which are covered by living coenosarc. The extremely low rate of exchange in this case is not entirely due to the isolating action of the living tissue sheet covering the skeleton: after the colonies were labelled with calcium-45 they were returned to the sea where they continued to grow, so that the radioactive layer eventually became buried under successive new layers of inactive skeletal material. Under these conditions the radioactive material was well protected from isotopic exchange which can occur only at the skeletal surface, in consequence of which no significant loss of calcium-45 could be observed.

The low exchangeability of already deposited calcium indicates that it does not significantly take part in physiological interactions with the overlying coral tissue. This is very different from what is observed in vertebrates where 25% to 35% of the bone calcium remains exchangeable with the calcium dissolved in the medium (*cf.*, for example, Dawson, 1955). The much greater exchangeability of the skeletal calcium in the vertebrates as compared with that of the corals is due to the fact that the bones of the former constitute an endogenous tissue system in dynamic equilibrium with the rest of the body constituents (Armstrong, 1955; Neuman and Neuman, 1953; Marshall, 1960), whereas the scleractinian corallum is an exoskeleton which, once formed, is entirely outside of the body proper, and its mineral contituents do not appear to be in steady-state equilibrium with the "milieu intérieur" of the polyps.

Summary and Conclusions

1. Equilibrium exchange of calcium between the skeleton and the medium was measured in dead and living corals under various conditions, using calcium-45 as isotopic tracer.

2. The calcium which is deposited as aragonitic carbonate into the skeleton of living corals is not in contact with the environment. Under the conditions of our experiments, no significant isotopic calcium exchange occurred in *Porites furcata* and *Millepora alcicornis* over a period of 23 days and 156 days, respectively.

3. In the dead parts of *Porites furcata* colonies where the corallum was exposed to direct contact with the medium, rapid isotopic exchange occurred so that the half-life of calcium-45 activity on the skeletal surface was only about five days.

4. In reef corals killed with formaldehyde, the amount of calcium-45 activity picked up by the skeleton from the medium in 48 hours by equilibrium exchange was the same in colonies in which the coenosarc had been preserved as in those from which all tissues had been removed, so that the naked corallum was in direct contact with the radioactive sea water. This indicates that, under the conditions of our experiments, the mechanical presence of the non-living coenosarc did not constitute a barrier to equilibrium exchange between the corallum and the environment.

5. In living corals in which the calcification rate was depressed to extremely low levels by simultaneous removal of the zooxanthellae and inhibition of carbonic anhydrase with 10^{-3} M acetazolamide, the amount of calcium-45 activity incorporated was 50% to 70% less than that exchanged by the dead control colonies from which all tissues had been removed by maceration. This indicates that the *living* coenosarc is an effective barrier to equilibrium exchange of calcium between the skeleton and the environment, even when the calcium deposition rate is almost zero.

6. Our experimental evidence indicates that the living coenosarc is fairly calcium-proof and prevents the skeleton from exchanging with the environment. Unlike vertebrates, in which a large fraction of the bone calcium remains in dynamic equilibrium with the dissolved calcium of the body fluids, the skeletal calcium of corals, once it is deposited into the corallum, probably does not take part in steady-state or equilibrium exchanges with the overlying tissues.

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RESPIRATORY REGULATION IN AMPHIBIAN DEVELOPMENT[1]

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There is now considerable evidence (reviewed by Slater and Hülsmann, 1959) that the respiratory rate of a cell abundantly supplied with oxidizable substrates is a function of the rate at which it metabolizes labile phosphorus compounds in response to energy demand. Consider, for example, the oxidation of glucose:

$$C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O.$$

Normally, this process is coupled to the esterification of inorganic phosphorus:

$$C_6H_{12}O_6 + 6\ O_2 + 38\ ADP + 38\ H_3PO_4 \rightarrow 6\ CO_2 + 44\ H_2O + 38\ ATP.$$

When work is to be done, stored ATP is utilized:

$$38\ ATP + 38\ H_2O \rightarrow 38\ ADP + 38\ H_3PO_4 + \text{Energy}.$$

As a result, the levels of ADP and inorganic phosphorus are raised momentarily, and respiration is quickened until ATP is restored. Thus, under normal conditions, the respiratory rate of a cell depends upon its rate of energy expenditure.

In the presence of uncoupling agents, *e.g.*, 2,4-dinitrophenol (DNP), the link between oxidation and phosphorylation is severed. When this happens, respiration proceeds at a rate limited only by the availability of oxidizable substrates, and without concurrent formation of ATP. At the same time, ATP-stores are depleted by destructive catalysis, and the ability to perform work deteriorates rapidly.

The burgeoning respiratory rates of developing embryos surely reflect ever-increasing expenditures of energy. Therefore, a study of the respiratory responses of developing embryos to uncoupling agents should yield important information about the energetics of development. Along these lines, a recent study of sea urchin embryos by Immers and Runnström (1960) has provided interesting data, and there is reason to believe that amphibian embryos are amenable to similar analysis. In the presence of DNP, explants of the tissues of frog gastrulae respire at twice the normal rate (Ornstein and Gregg, 1952); and, under similar conditions, intact gastrulae are partially depleted of their stores of esterified phosphorus and are prevented from undergoing further morphological change (Gregg and Kahlbrock, 1957).

In the work about to be reported, an uncoupling agent (DNP) has been used to study the development of respiratory regulation in *Rana pipiens* embryos, and also in hybrid embryos obtained by fertilizing *Rana pipiens* eggs with *Rana sylvatica* sperm. First studied by Moore (1946), these hybrids are incapable of developing

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beyond the early gastrula stage, and exhibit numerous other morphogenetic or metabolic anomalies (Gregg, 1957).

METHODS

Embryological

Developing embryos were obtained by stripping eggs from pituitary-injected *Rana pipiens* females into suspensions of active *Rana pipiens* or *Rana sylvatica* sperm. After two hours, they were dispersed thinly among fingerbowls and allowed to develop at temperatures ranging from 10° C. to 25° C. The medium, 10% Ringer's solution without phosphate or bicarbonate, was changed every two days, or more often. Before manometric measurements were made, watchmaker's forceps were used to free the embryos of their jelly coats.

Manometric

Respiratory rates were determined with a refrigerated Warburg respirometer equipped with 7-ml. single-side-arm center-well flasks. Carbon dioxide was absorbed on filter paper rolls placed in the center wells and saturated with 10% KOH. The flasks were shaken 75 complete cycles per minute at an amplitude of 6 cm. The temperature of the water bath was held constant at 24° C. Further details will be cited as the need arises.

Terminological

Developmental stages were determined by reference to the charts of Shumway (1940), which standardize the course of *Rana pipiens* development at 18° C. Therefore, regardless of their actual temperature histories, embryos in a given Shumway stage have been assigned the corresponding standard age at 18° C. Hybrid embryos have been assigned the same stages, and ages, as simultaneously developing *Rana pipiens* controls.

Respiratory rates are expressed in the following units: microliters of oxygen per hour per 50 embryos.

The respiratory rate exhibited by intact embryos at a given stage, and under standard conditions, is called the *respiratory norm* of embryos at that stage.

The respiratory rate exhibited by intact embryos at a given stage, and under maximal stimulation by DNP, is called the *respiratory potential* of embryos at that stage.

The quotient obtained by dividing the respiratory potential by the corresponding respiratory norm is called the *respiratory control quotient.*

RESULTS

The results obtained in the present work are now listed without commentary. They will be discussed in the next section.

(1) The respiratory norm of *Rana pipiens* embryos is a strongly increasing function of developmental age (Table I, Table III).

(2) The relation between the developmental age and respiratory norm of *Rana pipiens* embryos is best characterized as the exponential function consisting of all pairs (t, y) satisfying the following equations:

$$\text{(i)} \quad \begin{array}{ll} y = 5\,e^{0.026t} & (0 \leqslant t \leqslant 56) \\ y = 21\,e^{0.017(t-56)} & (56 \leqslant t \leqslant 140) \end{array}$$

TABLE I

Influence of 2,4-dinitrophenol on the respiratory rate of Rana pipiens embryos

Egg clutch	Stage	Age 18° C.	DNP-concentration, molar					
			Control	5×10^{-6}	10^{-5}	5×10^{-5}	10^{-4}	10^{-3}
A	11	34	11	19	24	33	31	—
	12½	46	18	28	35	40	35	1
	16	72	29	42	50	45	39	4
	19	118	56	93	104	85	79	25
B	6	7	6	6	13	24	23	3
	10½	30	12	16	22	32	28	2
	15	67	24	33	36	40	35	9
	10½	90	38	54	71	53	51	6
	19	118	56	85	102	71	64	26
	20	140	84	112	118	104	78	19

Main compartment of each flask: 20–50 embryos in 1 ml. 10% Ringer's. Side-arm of each flask: 0.5 ml. DNP in 10% Ringer's adjusted to give final concentration shown. Side-arm contents delivered to main compartment immediately after first reading. Readings were made for four or five hours (usually five) at half-hour intervals. Respiratory rates were constant after first hour. Entries designate average rates for last three (four) hours of four (five) hour runs.

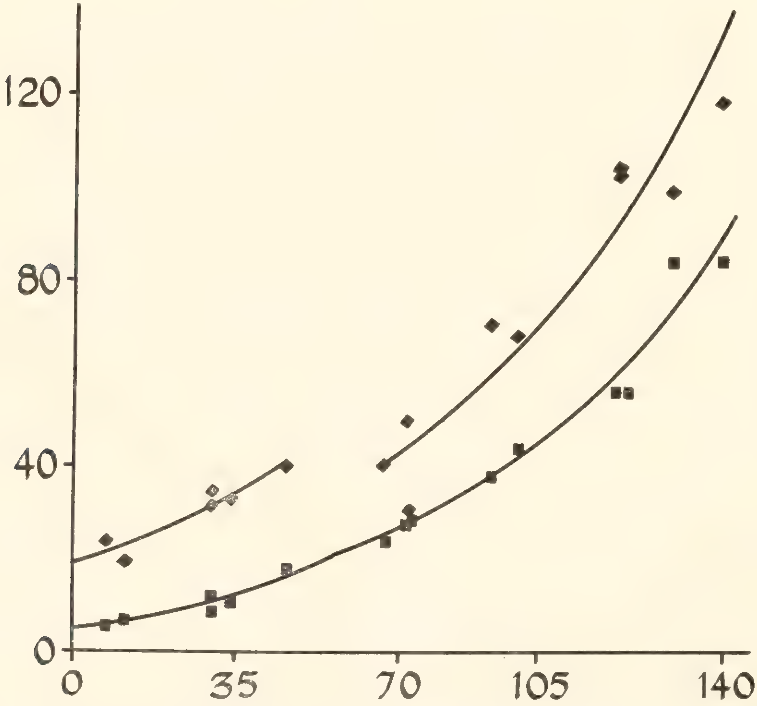

FIGURE 1. Respiratory norm (lower curve) and potential (upper curve) of *Rana pipiens* embryos. Abscissa, developmental age. Ordinate, respiratory rate.

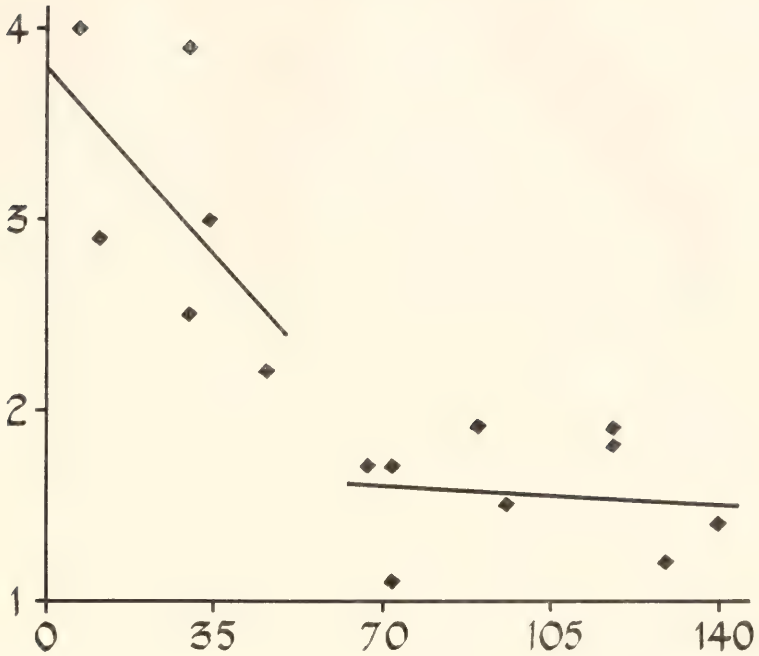

FIGURE 2. Respiratory control quotient, *Rana pipiens*. Abscissa, developmental age. Ordinate, respiratory control quotient.

TABLE II

Influence of 2,4-dinitrophenol on the respiratory rate of Rana pipiens ♀ × Rana sylvatica ♂ embryos

Egg clutch	Stage	Age 18° C.	DNP-concentration, molar					
			Control	5×10^{-6}	10^{-5}	5×10^{-5}	10^{-4}	5×10^{-4}
C	7½	11	5	13	20	22	18	20
	10½	30	3	10	18	31	30	17
	13½	56	3	13	21	28	26	15
	17½	90	12	18	22	29	—	20
	19	118	11	19	21	35	32	32
D	7^+	9	8	13	20	26	28	15
	11½	38	9	18	25	30	28	12
	15	67	13	17	22	35	37	19
	18	96	10	18	26	38	38	28

Fifty embryos per flask. DNP-treated embryos were equilibrated in DNP-solutions in 10% Ringer's for two hours preceding measurements. Readings were taken for one hour at 5-minute intervals.

Hybrid embryos do not develop beyond Stage 10: entries in the stage and age columns designate average developmental stages and corresponding ages of *Rana pipiens* control embryos.

where t is the developmental age and y is the respiratory norm (Fig. 1, lower curve).

(3) The respiratory activity of *Rana pipiens* embryos at any stage is stimulated by DNP in concentrations ranging from $5 \times 10^{-6}\ M$ to $1 \times 10^{-4}\ M$ (Table I, Table III).

(4) The respiratory potential of *Rana pipiens* pre-neurulae is exhibited under treatment with DNP at concentrations near $5 \times 10^{-5}\ M$; that of neurulae and older embryos is exhibited under treatment with DNP at concentrations near $10^{-5}\ M$ (Table I, Table III).

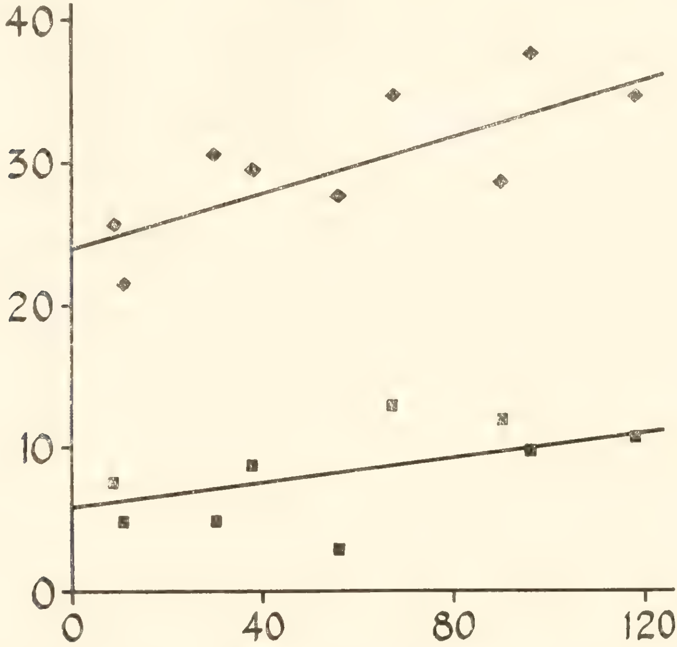

FIGURE 3. Respiratory norm (lower curve) and potential (upper curve) of *Rana pipiens* ♀ × *Rana sylvatica* ♂ embryos. Abscissa, developmental age. Ordinate, respiratory rate. (24° C.)

(5) The relation between the developmental age and respiratory potential of *Rana pipiens* embryos is best characterized as the exponential function consisting of all pairs (t, y) satisfying the following equations:

$$\text{(ii)} \quad \begin{array}{ll} y = 19\,e^{0.017t} & (0 \leqslant t \leqslant 46) \\ y = 41\,e^{0.016\,(t-67)} & (67 \leqslant t \leqslant 140) \end{array}$$

where t is the developmental age and y is the respiratory potential (Fig. 1, upper curve). For reasons explained later, the respiratory potentials corresponding to some values of t $(46 < t < 67)$ are left undefined.

(6) The respiratory control quotient of *Rana pipiens* embryos decreases rapidly from 3.8 at fertilization to 2.5 at 46 hours, and slowly from 1.6 at 67 hours to 1.5 at 140 hours (Fig. 2). For reasons explained later, the respiratory control quotients of embryos between the ages of 46 hours and 67 hours are left undefined.

(7) The respiratory norm of hybrid embryos is a weakly increasing function of developmental age (Table II).

(8) The relation between the developmental age and respiratory norm of hybrid embryos is best characterized as the linear function consisting of all pairs (t, y) satisfying the following equation:

(iii) $$y = 6 + 0.045t \qquad (0 \leqslant t \leqslant 118)$$

where t is the developmental age and y is the respiratory norm (Fig. 3, lower curve).

(9) The respiratory activity of hybrid embryos at any stage is stimulated by DNP in concentrations ranging from $5 \times 10^{-6}\,M$ to $5 \times 10^{-4}\,M$ (Table II).

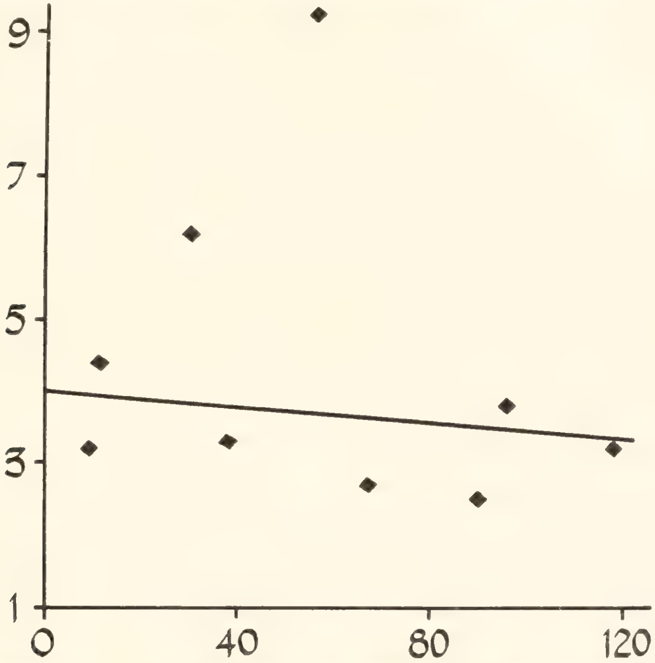

FIGURE 4. Respiratory control quotient, *Rana pipiens* ♀ × *Rana sylvatica* ♂. Abscissa, developmental age. Ordinate, respiratory control quotient.

(10) The respiratory potential of hybrid embryos is exhibited under treatment with DNP at concentrations near $5 \times 10^{-5}\,M$ (Table II).

(11) The relation between the developmental age and respiratory potential of hybrid embryos is best characterized as the linear function consisting of all pairs (t, y) satisfying the following equation:

(iv) $$y = 24 + 0.10t \qquad (0 \leqslant t \leqslant 118)$$

where t is the developmental age and y is the respiratory potential (Fig. 3, upper curve).

(12) The respiratory control quotient of hybrid embryos decreases from 4 at fertilization to 3.3 at 118 hours (Fig. 4).

(13) Homogenization in buffer-saline solution has little effect upon the respiratory activity of young *Rana pipiens* embryos, but it powerfully stimulates the respiratory activity of older ones (Table III).

(14) The respiratory rates exhibited by homogenized *Rana pipiens* embryos are not affected by DNP in concentrations ranging from 10^{-5} *M* to 5×10^{-5} *M* (Table III).

TABLE III

Influence of 2,4-dinitrophenol on the respiratory rate of intact embryos and cell-free homogenates (Rana pipiens)

Egg clutch	Stage	Age 18° C.	DNP-concentration, molar		
			Control	10^{-5}	5×10^{-5}
E Intact	7½	11	7	—	20
	10½	30	9	—	35
	16	72	28	31	—
	18	96	44	68	—
	19½	129	84	99	—
E Homogenized	7½	11	8	6	9
	10½	30	8	10	7
	16	72	132	130	129
	18	96	132	128	124
	19½	129	146	136	137

Intact embryos. Fifty embryos per flask. DNP-treated embryos were equilibrated in DNP solutions (in 10% Ringer's) for one to two hours preceding measurements. Readings were taken for one hour at 5-minute intervals.

Homogenates. Embryos suspended in ice-cold 10^{-2} *M* phosphate buffer made up in 0.065% NaCl (50 embryos per ml.) were homogenized with a Lourdes homogenizer. Aliquots (1 ml.) were transferred to respirometer flasks. Immediately after first reading, DNP solutions (0.5 ml. in buffer-saline, "pH 7.48") were tipped into main compartments to give final DNP-concentrations desired. Readings were taken for one hour at 5-minute intervals.

Entries in the stage and age columns designate average developmental stages and corresponding ages.

DISCUSSION

The results listed in the previous section will now be discussed, in sequence.

(1) Embryologists have known for a long time that developing amphibian embryos are characterized by waxing respiratory norms. Our first result, then, is not new, and our chief concern will be to find an explanation for it.

One explanation is provided by the assumption that developmentally increasing respiratory rate is the direct result of the synthesis of respiratory machinery. But recent experiments (Spiegelman and Steinbach, 1945; Gregg and Ray, 1957) have established that homogenates of newly fertilized eggs can be made to respire endogenous substrates at rates exceeding those of intact embryos at any stage of development. From the outset, therefore, there is more than enough respiratory apparatus to support any rate of respiration normally exhibited by a developing embryo, and the suggested explanation cannot be correct.

Another explanation, sponsored by Spiegelman and Steinbach, and also by Gregg and Ray, assumes that increasing respiratory norms are the direct result of processes progressively facilitating effective contact between respiratory enzymes and their substrates, *i.e.*, that the respiratory rate at any stage is limited simply by the rate at which some respiratory enzyme is able to combine with its substrate. But, under this assumption, we are left without good reason to expect an elevation of respiratory rate in the presence of uncoupling agents, and it is best abandoned.

Still another explanation (the one we shall adopt here) is provided by the theory outlined in the introduction. According to this theory, the respiratory norm is a function of the rate of energy expenditure; and waxing respiratory norms thus are to be ascribed to waxing rates of energy expenditure. In this connection it is known that developing *Rana pipiens* embryos are characterized by increasing rates of carbohydrate utilization (Gregg, 1948), and by increasing rates of turnover of labile phosphorus (Kutsky, 1950).

(2) Our mathematical analysis of the relation between developmental age and respiratory norm (*Rana pipiens*) has been made following the precedent established by Atlas (1938) and by Moog (1944), and the results of it are in considerable agreement with theirs. The following version of their equations is obtained by changing units to correspond with those used in the present investigation and by confining values of t to the interval (0–140):

$$\text{(v) Atlas} \quad \begin{aligned} y &= 3\,\mathrm{e}^{0.038t} && (0 \leqslant t \leqslant 62) \\ y &= 16\,\mathrm{e}^{0.019\,(t-62)} && (62 \leqslant t \leqslant 140) \end{aligned}$$

$$\text{(vi) Moog} \quad \begin{aligned} y &= 5\,\mathrm{e}^{0.077t} && (0 \leqslant t \leqslant 50) \\ y &= 21\,\mathrm{e}^{0.031\,(t-50)} && (50 \leqslant t \leqslant 140) \end{aligned}$$

Considering the nature of the supporting data, (i), (v) and (vi) are in good agreement. For *Rana pipiens* embryos, therefore, it appears to be established that respiratory norm is an exponential function of developmental age, and that respiratory acceleration decreases at some point in the age interval (50–62). For possible explanations of the acceleratory change, readers are referred to the papers of Atlas and Moog.

(3) We have already mentioned that *Rana pipiens* embryos are well-provisioned with respiratory substrates; therefore, on the basis of the theory outlined in the introduction, the stimulatory effect of DNP is to be expected.

(4) What is unexpected is the finding that the concentration of DNP eliciting the respiratory potential of *Rana pipiens* embryos is five times as great for the age interval (0–67) as for the age interval (72–140). There is no parallel for it in the development of sea urchin embryos, whose respiration is maximally stimulated at any stage by $5 \times 10^{-5}\,M$ DNP (Immers and Runnström, 1960); and, pending further investigation, it remains unexplained.

(5) There is no precedent to guide mathematical analysis of the data relating developmental age to respiratory potential (*Rana pipiens*); and subsequent investigation may necessitate revision of equations (iv), which have been obtained by taking the data at face value. For what it is worth, Figure 1 (upper curve) shows that the development of the respiratory potential of *Rana pipiens* embryos proceeds in three phases: two of exponentially increasing potential, separated by one whose characteristics are not known. The second phase may be one of constant potential,

as the data suggest; or, if the first and third phases are of greater duration than shown, it may be one of abruptly decreasing potential; or, although there is no reason for so believing, later work may show that the first and third phases actually intersect in the age interval (46–67), thus abolishing the second phase entirely. We shall leave the question open, after noting that Immers and Runnström (1960) observed a transient decline of respiratory potential in sea urchin embryos entering the mesenchyme blastula stage. But their result, also, was reported in a mood of skepticism.

In any case, it is clear that the respiratory potential of *Rana pipiens* embryos increases with age, remaining well above the respiratory norm, and thus maintaining a considerable margin of safety for energy expenditure. We have accounted for the developmental increase of respiratory norm by supposing that it is a function of increasing rate of energy expenditure. To account for increasing respiratory potential, it is necessary to assume the occurrence of intracellular structural changes progressively enhancing contact between respiratory enzymes and substrates. Data bearing upon this assumption are neither crucial nor consistent. Weber and Boell (1955) have found that the specific activity of mitochondrial cytochrome oxidase is an increasing function of developmental age (*Xenopus laevis*), thus indicating some process of mitochondrial differentiation; on the other hand, Spiegelman and Steinbach (1945) were unable to observe any developmental increase of the cytochrome oxidase activity of homogenates (*Rana pipiens*). Nevertheless, our assumption is supported by the electron microscopical study of Eakin and Lehmann (1957), who discovered profound developmental alterations of structural complexity and localization of the intracellular components, including mitochondria, of the ectoderm of neurulating amphibian embryos (*Xenopus laevis, Triton alpestris*). Therefore, until better assumptions are available, we shall adhere to our present one.

(6) The respiratory control quotient is a convenient measure of the degree to which the rate of energy expenditure holds the respiratory norm below the respiratory potential. Figure 1 shows that, during the first 46 hours of development, energy expenditure in *Rana pipiens* embryos is such as to permit a rapid approach of the respiratory norm to the respiratory potential; from 67 hours on, the relation is nearly stabilized, and respiratory norm is practically a constant fraction of respiratory potential. For reasons stated in the discussion of result (5), respiratory control quotients corresponding to the age interval (46–67) are left undefined. It is worth noting that a similar relation between respiratory norm and respiratory potential characterizes the development of sea urchin embryos (Immers and Runnström, 1960).

(7) We come now to the respiration of hybrid embryos. In a general way the data agree with those of Barth (1946) in showing that the respiratory norms of such embryos become increasingly subnormal as time goes on, and the same may be said of the rates at which they utilize carbohydrate reserves (Gregg, 1948). It appears, therefore, that they expend energy at increasingly subnormal rates; and this, on the theory of respiratory control we are adopting, is the reason for their progressively subnormal respiratory norms. On this basis, we should expect to find increasingly subnormal rates of turnover in their pools of labile phosphorus, but data are not yet available. There may be nothing wrong with their respiratory machinery, for their

homogenates respire at rates quantitatively similar to those of homogenates of *Rana pipiens* control embryos (Gregg and Ray, 1957).

(8) Our mathematical analysis of the relation between the developmental age and respiratory norm of hybrid embryos is based upon the assumption of linearity. The more precise data of Barth (1946) suggest that this assumption is not quite correct, but it is a useful approximation to the exact state of affairs. It should be noted that the intercept 6 of equation (iii) is in good agreement with the intercept 5 of equation (i).

(9) The view that the respiratory machinery of hybrid embryos may be entirely normal is supported by the finding that their respiratory rates are stimulated from 300 to 400% by $5 \times 10^{-5}\,M$ DNP; for no better response is obtainable from *Rana pipiens* control embryos.

(10) This result need not be elaborated, except by pointing out that the concentration of DNP eliciting the respiratory potential of hybrid embryos at any stage is the same as that eliciting the respiratory potential of *Rana pipiens* pre-neurulae: there is no developmental shift in sensitivity to DNP like that exhibited by neurulating *Rana pipiens* embryos.

(11) On the assumption of linearity, the rate at which the respiratory potential of hybrid embryos increases is given by equation (iv). The increase of respiratory potential, though slower than normal, still is unnecessary; for the respiratory norms of hybrid embryos never overtake their initial respiratory potential (Fig. 2). Nevertheless, an increase of respiratory potential occurs; and to explain it we shall assume, in accordance with the discussion of result (5), that intracellular changes facilitating respiratory enzyme-substrate union take place in hybrid embryos as well as in normal ones, though at a much slower rate. Electron microscopical and biochemical studies of the intracellular particulates of hybrid embryos are much needed.

(12) This result does not require further commentary: reference to the discussion of result (6) will make its interpretation perfectly evident.

(13) This finding confirms the work of Gregg and Ray (1957): unless they are treated with a detergent (*e.g.*, deoxycholic acid), homogenates of very young *Rana pipiens* embryos do not respire at rates much different from the respiratory norm; but the respiratory rates of homogenates of progressively older embryos rapidly exceed the respiratory norm. We shall return to this topic in the discussion of the last result.

(14) The failure of DNP to elevate the respiratory activity of homogenates of *Rana pipiens* embryos at any stage of development is extremely puzzling, and no adequate explanation for it is now at hand.

Homogenates of adult tissues frequently do not exhibit a respiratory response to the presence of uncoupling agents. In such cases, respiratory rate appears to be limited, not by the rate of turnover of labile phosphorus, but by the low availability of readily oxidizable substrates; generally, this limitation is overcome by adding pyruvate, succinate or other respiratory metabolites (Krebs, 1959). This account of the matter is not applicable to homogenates of amphibian embryos (see the discussion of result (1)).

An arbitrary explanation, for which there is little independent support, may be constructed along the following lines. First, let us suppose that homogenization, by activating ATP-ase, results in some maximum elevation of the levels of ADP and inorganic phosphorus. Second, let us suppose that homogenization is accompanied by the envelopment of respiratory particulates in lipo-protein or other envelopes. Third, let us suppose that the degree of envelopment decreases with developmental age. The first assumption guarantees a high rate of respiratory activity, other conditions permitting; and explains why DNP is without effect, the levels of ADP and inorganic phosphorus already being maximum. The second assumption explains why the respiratory activity of homogenates of young embryos is low, for we may expect that respiration under these conditions will be limited by the rate at which respiratory substrates are able to penetrate lipo-protein barriers. It also explains why the respiratory rate of homogenates of young embryos is elevated by detergents, for these may be expected to disperse lipo-protein deposits around respiratory particulates; or, even, to fragment those particulates (Siekevitz and Watson, 1956). The third assumption explains why the respiratory activity of homogenates is an increasing function of developmental age.

Summary

1. The respiratory rate of *Rana pipiens* control embryos is an increasing function of developmental stage, with an acceleratory change at the onset of the formation of the neural folds.

2. At any stage of development, the respiratory rate of *Rana pipiens* embryos is elevated by the presence of 2,4-dinitrophenol (DNP). The degree of stimulation obtainable ranges from about 400% of the control rate at the beginning of development to about 150% of the control rate at the gill-circulation stage.

3. The respiratory rate of *Rana pipiens* ♀ × *Rana sylvatica* ♂ embryos is an increasing function of time, but the rate of increase is very much lower than that of the respiratory rate of *Rana pipiens* controls.

4. At any stage, the respiratory rate of hybrid embryos is elevated by DNP. The degree of stimulation obtainable ranges from about 400% at the beginning of development to about 300% at 118 hours after fertilization (18° C.).

5. The respiratory activity of homogenates of *Rana pipiens* embryos at any stage is not altered by the addition of DNP.

6. The relevance of these findings to the question of embryonic respiratory control is discussed. It is concluded that, within the capacity to respire, respiration is governed by energy expenditure, and that the capacity to respire increases with age as the result of intracellular changes facilitating contact between respiratory enzymes and substrates.

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THE INFLUENCE OF SALINITY ON THE MAGNESIUM AND WATER FLUXES OF A CRAB

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The shore crab *Pachygrapsus crassipes* is known to be an osmotic regulator in both dilute and concentrated sea water, but its antennary glands are ineffective as organs of osmo-regulation inasmuch as the urine remains essentially isotonic to the blood, regardless of the salinity of the external medium (Jones, 1941; Prosser *et al.*, 1955; Gross, 1957a). On the other hand, Prosser *et al.* (1955) demonstrated Mg concentrations in the urine of this species when it is exposed to osmotic stresses, which suggests that the antennary glands are effective regulators of Mg. Gross (1959) confirmed these observations, but pointed out that the volume of urine produced, as well as the urine concentration of Mg, must be known before the antennary glands could be considered certain regulators of this cation. Prosser *et al.* (1955) also observed that while the urine concentration of Mg increased tremendously when the crab was immersed in increasingly saline media, the urine Na, contrary to expectation, decreased. They suggested that Na and Mg compete for transport across the membranes of the antennary gland with Mg predominating. When the crab was immersed in artificial Mg-free sea water equivalent to 170% of normal salinities, the observed concentration of Na in the urine was much higher than it was when the crab was immersed in 170% natural sea water, thus supporting the suggestion. However, the effects of such a treatment on the urine Mg concentration were not reported.

Even though *Pachygrapsus* is a strong regulator in large osmotic stresses, its blood tends toward concentrations which are intermediate between those it has in normal sea water and the concentration of the external medium (Jones, 1941; Prosser *et al.*, 1955; Gross, 1957a). Gross (1957a) demonstrated that such changes in the concentration of the blood are brought about by salts and not water; that is, the volume changes of the animal were insignificant. This must mean that either the formed tissues when bathed by such altered blood concentrations also remain unchanged in volume or that their volumes change at the expense of the blood space. Shaw (1955) demonstrated that the volume of muscle tissue in the hyper-regulating crab *Carcinus* increased when the animal was immersed in dilute sea water.

The present investigation will show that the efflux of Mg from *Pachygrapsus* is principally a function of water turnover and not due immediately to the Mg gradient between blood and external medium. It also will be shown that muscle tissue increases in volume when the crab is immersed in dilute sea water, and that it decreases in volume when the crab is immersed in concentrated sea water. This results in volume alterations in the blood space.

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MATERIAL AND METHODS

The lined shore crab, *Pachygrapsus crassipes* Randall, was collected at Dana Point, Laguna, and Ballona Creek, California. Only mature crabs of more than 15 grams were used. Care was taken that none was undergoing moult.

Blood and urine were sampled as previously described; analyses of Na and K were made by flame photometry and Ca and Mg by titration with ethylene diamine tetra acetic acid (EDTA) (Gross, 1959).

Artificial sea water was prepared according to the tables of Barnes (1954), normal sea water being considered to contain the following concentrations (meq./l.) of the major ions: Na, 460; K, 10; Ca, 20; Mg, 104; Cl, 538; SO_4, 56. The pH was adjusted to 8.0. In the Mg-free media Na was substituted for the deleted Mg and where Mg was increased above normal, relative to the other ions, Na was deleted accordingly to attain the desired osmotic pressure.

The effect of abnormal concentrations of medium Mg on blood and urine ionic concentrations was studied in two ways: (1) Groups of crabs from 100% natural sea water were immersed into small volumes of Mg-free artificial sea water of salinities equivalent to 50%, 100% and 150% of natural sea water, and into media equivalent to 50% in salinity but containing Mg equal to that of 100% natural sea water (104 meq./l.). Also, one group was immersed in a medium equivalent to 100% sea water in total salinity, but which contained half again as much Mg (156 meq./l.). All animals first were rinsed in the respective test medium before immersion. After 24 hours, the blood and urine of the experimental animals were analyzed for Na, K, Ca and Mg; the media which were originally Mg-free were analyzed for Mg. In the small volumes of media used for this group, the crabs could rise partially out of the water. This kept the mortality rate low over the 24-hour period of immersion, thus permitting a study of ionic alterations in the blood and urine in the artificial media.

(2) The second group of experiments was conducted primarily to measure rates of Mg excretion. Here animals were placed in large volumes of medium to assure complete and uniform immersion throughout the test period. Crabs previously acclimatized for 24 hours in natural sea water of salinities respective to their subsequent test media were immersed in large volumes of Mg-free 50%, 100% and 150% artificial sea water, these, again, having been rinsed first in the test media. During acclimatization in natural sea water, the crabs could rise out of the water. Again after a period of immersion ranging from one to six hours the blood and urine of the animals were analyzed for Na, K, Ca and Mg and the medium was analyzed for Mg. All experiments were conducted in a temperature-controlled room at 15° C.

In order to determine the volume changes in the muscle tissue of *Pachygrapsus* under different osmotic stresses, leg muscle from animals which had been immersed for three days in 50%, 100% and 150% natural sea water was rinsed in isotonic glucose and blotted uniformly. (Glucose concentrations were calculated from the tables in Gross, 1959.) These were then weighed and dried to constant weight in a drying oven at 95° C. The difference between dry weight and wet weight was considered to be the water content of the tissue.

Changes in the blood space volume were shown by calculating the volume of distribution of C^{14}-tagged sucrose in the blood space one minute after injection. Crabs removed from immersion for three days in 50%, 100% and 150% natural sea water were injected at the base of the fourth walking leg with 0.3 to 0.5 ml. (depending on the size of the crab) of 1 *M* sucrose which had been tagged with C^{14}.

About one minute after the injection, a sample of blood was taken from the opposite side of the animal and diluted with 5 ml. of 1 *M* untagged sucrose. The quantity of blood was determined by weight and averaged about 0.3 gram. Then a 0.10-ml. aliquot of the diluted blood was absorbed onto a filter paper disc, allowed to dry and counted on a Nuclear-Chicago scaling unit. Mean counts thus obtained for three discs made from each diluted blood sample were compared with the mean counts of three 0.10-ml. aliquots of the dose which were plated in the same manner. Since the blood was diluted in 1 *M* sucrose and the dose was 1 *M* sucrose, the error due to self-absorption should be essentially the same in radio-assays of both blood and dose. Care was taken to assure uniform geometry. At least 1000 counts were observed for each sample, and maximum rates did not allow significant coincidence.

TABLE I

Ion concentration in the urine of Pachygrapsus *immersed in small volumes of artificial sea water*

		50%			100%			150%		
		Mean	S.D.	No.	Mean	S.D.	No.	Mean	S.D.	No.
Na (meq./l.)	N	380	60	37	378	64	15	353	106	30
	0	400	28	12	332	96	12	430	81	15
	+	401	33	25	319	80	8			
K (meq./l.)	N	10.0	3.5	37	7.8	1.4	15	9.6	1.1	30
	0	14.3	8.7	12	15.9	8.0	12	16.0	4.2	15
	+	11.0	3.8	25	7.0	2.0	8			
Ca (meq./l.)	N	32.7	7.1	31	36.0	6.3	15	47.9	5.2	20
	0	34.5	4.5	13	34.5	4.5	13	47.6	18.6	15
	+	31.1	8.7	19	37.0	7.8	14			
Mg (meq./l.)	N	70.5	41	31	305	130	15	408	122	29
	0	85.0	42	16	298	104	13	281	85	15
	+	65.5	23	18	368	101	14			

N = crabs immersed in concentrations of natural sea water.
0 = crabs immersed in Mg-free sea water.
+ = crabs immersed in artificial sea water containing abnormally high Mg: in 50% sea water = 104 meq./l.; in 100% sea water = 156 meq./l.

The volume of distribution for sucrose in one minute was, therefore, calculated from the equation: $V = \frac{d/b}{w} \times 100$

where V = volume of distribution of sucrose in one minute (% body weight);
d = observed total activity (counts/min.) injected into the crab;
b = observed activity (counts/min.) per gram of blood;
w = weight of crab (g.).

Also, a correction was applied for the volume of the dose.

After the blood samples were taken, the crabs were placed in a closed chamber containing a $Ba(OH)_2$ trap designed to absorb CO_2. Radio-assay of the total precipitate thus collected in 24 hours for ten crabs demonstrated no significant activity, suggesting that the sucrose was not metabolized. It is possible that some was fixed in the body, but it seems more likely that it remained in solution in an unchanged state at least for the brief period (one minute) during which the dilution was being observed.

RESULTS

Table I compares the urine Mg of crabs immersed in small volumes of artificial sea water with the urine Mg of crabs immersed for 24 hours in small volumes of natural sea water. The value for crabs in 100% natural sea water is higher than previously reported, but is believed to be more reliable because of improved technique in sampling the urine; the values for crabs in 50% and 150% natural sea water have been reported previously (Gross, 1959). It is clear from these data that when *Pachygrapsus* is immersed in 50% and 100% Mg-free artificial sea water, the concentration of Mg in its urine is close to the concentration of Mg in the urine of crabs immersed in the same salinities of natural sea water. When a crab is immersed in 50% sea water for 24 hours, the Mg concentration of its urine is about the same whether the medium Mg is 0, 52 or 104 meq./l. In salinities of 100% normal, the observed mean concentration for urine Mg was about the same for animals immersed in Mg-free water as for those from natural sea water; for animals immersed in 100% artificial sea water containing abnormally high Mg, the urine Mg was slightly higher than for crabs from 100% natural sea water (368 meq./l. to 314 meq./l., respectively), but these means are not significantly different (Table I). The urine Mg of crabs immersed in small volumes of Mg-free 150% sea water was less than that for crabs from 100% or 150% natural sea water. This is difficult to interpret, but possibly could be explained as a reflection of the crab's greater tendency to remain out of the artificial medium. Such an argument is supported by the fact that the urine Mg for crabs immersed in large volumes of Mg-free 150% sea water averaged about the same (470 meq./l.) as did crabs from small volumes of 150% natural sea water (408 meq./l.). Also, when the crabs were immersed in large volumes of 100% Mg-free sea water, the urine Mg was about the same as for crabs immersed

TABLE II

Excretion of Mg by Pachygrapsus *in stress media*

Test medium	Medium volume*	Mean urine Mg meq./l.	S.D.	No.	Mean Mg excreted meq./day/g.	S.D.	No.
150% sea water without Mg	small (24 hrs.)	281	85	15	0.0021	0.001	14
	large (6 hrs.)	471	152	17	0.0073	0.0054	18
100% sea water without Mg	small (24 hrs.)	298	104	13	0.0070	.0033	14
	large (6 hrs.)	243	103	15	0.0095	0.0084	15
50% sea water without Mg	small (24 hrs.)	85.0	42	16	0.0066	0.0030	20
	large (1 hr.)	72.0	44	12	0.0433	0.022	12

* Time in parentheses = period of immersion.

in 100% natural sea water (Tables I and II). Table III presents probability values for analyses of Mg excretion.

It might be argued that the animals immersed in the larger volumes of artificial sea water were previously acclimatized to the respective salinities, and that sufficient time had not been given to permit alteration of the urine concentrations. However, when 13 crabs were transferred directly from 100% natural sea water and immersed for six hours in a large volume of 150% Mg-free sea water, the mean urine Mg was 713 meq./l., indicating not only that considerable changes can occur in the urine Mg concentration in six hours, but also that the greater hyperosmotic stress due to transferring the animals directly from 100% natural sea water to 150% artificial sea water probably made the urine even more concentrated with respect to Mg, again independently of the influx of this ion from the medium. Thus, for the periods indicated the concentration of Mg in the urine is not determined by the influx of this ion from medium, but rather, at least indirectly, by the osmotic pressure of the external medium.

TABLE III

Probability values for analyses of Mg excretion

A. Comparison of urine Mg concentrations: crabs immersed in artificial sea water vs. crabs immersed in natural sea water

	50% Sea water		100% Sea water		150% Sea water	
	Large volume	Small volume	Large volume	Small volume	Large volume	Small volume
Mg-free	>0.50	>0.20	=0.20	>0.50	>0.10	<0.001
Excess Mg		>0.50		>0.20		

B. Comparison of rates of Mg loss by crabs completely immersed in Mg-free artificial sea water

100% S.W. vs. 150% S.W.	>0.30
100% S.W. vs. 50% S.W.	<0.001
150% S.W. vs. 50% S.W.	<0.001

Table I also compares the urine concentrations of the other three major cations of the crabs immersed in small volumes of artificial sea water with those immersed in the respective salinities of natural sea water. Contrary to the findings of Prosser *et al.* (1955), there is no dramatic increase in urine Na when Mg is deleted from the medium. Although in 100% artificial sea water with high Mg the urine Na of *Pachygrapsus* was somewhat low, this could be accounted for by the low Na in the medium rather than the high Mg. Then in 150% Mg-free sea water the urine Na was high, but again this was likely due to the high Na in the medium, substituting for the deleted Mg. The concentration of Ca in the urine also seems unaffected by the absence or relative increase of Mg. With regard to K, the urine concentrations of this ion are significantly higher when the animal is immersed in 100% and 150% Mg-free sea water, than when immersed in the same concentrations of natural sea water ($P < 0.01$). The mean urine K for animals immersed in 50% Mg-free sea water also was higher than for crabs immersed in 50% natural sea water, but the difference cannot be shown to be significant. Neither is the urine K of animals immersed in 50% sea water with normal Mg concentrations (104 meq./l.) significantly different from that of crabs from 50% natural sea water.

Table II reveals the rate of Mg excretion in the different Mg-free salinities. Thus, considering only those crabs immersed in the large volume where they could not rise out of the water, it can be seen that the mean Mg excreted in 100% sea water is greater than the mean Mg excretion in 150% sea water, but the difference between these means is not significant (Table III). On the other hand the rate of Mg loss is four times as great in 50% sea water as it is in normal sea water; this difference is highly significant, $P < 0.001$ (Table III). Table II also shows that less Mg is lost to a small medium than to a large one. This, of course, would be expected because the animals could rise out of the small volume. Among all test media the difference in rates of Mg excretion between large and small volume treatments was smallest for 100% sea water, suggesting that in this salinity there is a minimum attempt to rise out of the water. The tendency for this crab to avoid an osmotic stress has been noted previously (Gross, 1957b).

The concentration of Mg in the urine of crabs immersed in large volumes of artificial sea water also is given in Table II. It will be recalled that these crabs first were acclimatized to the respective salinities of natural sea water before treatment in artificial sea water. Thus, there was no large change in the osmotic gradient to which the animal was subjected following acclimatization. The indicated periods of immersion were chosen because after such time in the test media, the urine Mg did not differ significantly from that of crabs removed from the respective acclimatizing salinities of natural sea water. Immersion periods of more than one hour in large volumes of Mg-free 50% sea water apparently deplete the Mg supply of the crab and the urine Mg becomes greatly reduced in concentration. Mg excretion of *Pachygrapsus* immersed for one hour in all three salinities of Mg-free sea water could not be compared because in this brief time insufficient amounts of the ion were released in 100% or 150% sea water to be detected with precision by the methods available.

If, then, the consistent concentration of Mg in the urine were known throughout a period of immersion and the amount of Mg lost to the medium in that period also were known, then assuming that the antennary glands are the sole pathways of Mg efflux, the volume of urine necessary to excrete the observed loss of Mg can be calculated. Estimations of urine flow from mean rates of Mg loss and mean Mg concentrations in the urine follow: in 150% sea water the volume of urine production was calculated to be 1.5% body weight/day; in 100% sea water, 3.9% body weight/day and in 50% sea water, 58% body weight/day.

The value for *Pachygrapsus* immersed in 100% sea water compares favorably with values reported by Webb (1940) on *Carcinus* and by Robertson (1939) for *Cancer* when the crabs were immersed in normal sea water, but only half the value reported by Nagel (1934) for *Carcinus* immersed in normal sea water. These workers, however, plugged the nephropores and assumed the gain in weight was due to urine which could not escape. Values obtained by three different methods on the prawn, *Palaemonetes*, in 100% sea water were more than twice as large as the above rate for *Pachygrapsus* (Parry, 1955).

As might be expected, the calculated rate of urine flow for *Pachygrapsus* was less in 150% sea water than in 100% sea water and the rate in 50% sea water greater than in normal sea water, but of such magnitude (58% body weight/day) that it is subject to question. This rate was determined on the basis of Mg excretion after one hour total immersion, but since the crabs had been acclimatized to 50% natural sea water before treatment in the Mg-free medium, there was no large increase in the osmotic gradient between external medium and the blood of the animal. However, forced total immersion, which did not take place in the acclimatizing procedure, caused more surface of the crab to be exposed to stress and this probably caused an increase in the water influx and the consequent increase in urine flow. It should be emphasized again that the concentration of Mg in the urine following immersion in large volumes of 50% Mg-free sea water averaged about the same as for crabs immersed for 24 hours in 50% natural sea water. It is thus likely that the urine Mg concentration for this group of crabs did not change during the one-hour immersion period. Either, then, the rate of urine flow for crabs thus treated is as calculated (58% body weight/day) during that period of immersion, or in such a hypotonic medium, mechanisms of Mg loss are different from those in crabs immersed in 100% and 150% Mg-free sea water. The Mg gradient between blood and external medium (Mg-free) would be about the same for all three conditions; yet Table II demonstrates the greatest mean loss to 50% and the smallest mean loss to 150% sea water. It is our opinion that if the principal pathway for Mg loss in all the above conditions were the antennary glands, then the above value for the rate of urine flow is a fair approximation for the conditions described. It would follow that such a rate could not be sustained, and it is interesting that the number of fatalities for crabs immersed for one hour in the large volume of 50% sea water was twice as large as the combined number of fatalities for crabs totally immersed for six hours in 100% and 150% sea water. Also, urine Mg in a few crabs which survived for six hours in 50% Mg-free sea water was essentially nil, and the actual amount of Mg lost to the medium was about twice that lost by crabs immersed in large volumes of Mg-free 100% sea water for the same period. This indicates exhaustion of the Mg supply in the crabs. When nine crabs which had been acclimated for 24 hours in small volumes of 50% natural sea water were transferred to large volumes of the same medium, for six hours, the mean urine Mg dropped to 47.2 meq./l., S.D. = 13.4. This is significantly less than the urine Mg of crabs from small volumes of 50% natural sea water (Table I): $P = 0.01$. It is suggested that sudden total immersion in natural 50% sea water causes a depletion of Mg reserves more rapidly than they can be replenished from a medium of this salinity. The low mortality rate in this group of crabs also suggests that Mg depletion is a cause of death in the crabs immersed in Mg-free 50% sea water, but also raises the question as to how long *Pachygrapsus* can survive totally immersed in 50% sea water in nature.

In order to estimate the urine flow when *Pachygrapsus* was removed from water, ten crabs were taken from normal sea water, rinsed in distilled water to wash away residual salts, then blotted dry. The crabs then were placed in dry containers and kept in a relatively humid temperature-controlled room at 15° C. for 72 hours. Then the crabs and their containers were rinsed with distilled water and the washings saved for Mg analysis. Also, the urine from these animals was sampled and analyzed for Mg. The urine concentration for Mg and the total excretion of Mg into the container then should yield the volume of urine flow during the 72-hour period. The average calculated rate of urine produced, thus determined, was 0.02% body weight/day, which is hardly significant.

Table IV demonstrates the effects of altered Mg in the medium on the blood concentration of the four major cations in *Pachygrapsus*. As would be expected, the blood Mg concentrations of crabs immersed in 100% and 150% Mg-free sea water are less than those of crabs from the same salinities of natural sea water. However, the blood Mg of crabs immersed in Mg-free 50% sea water was not significantly less than the blood Mg for crabs from 50% natural sea water. On the other hand, blood Mg for crabs immersed in 50% artificial sea water which contained normal Mg (104 meq./l.) was about equal to the blood Mg of crabs from 100% natural sea water (20 meq./l.). Also, the mean blood Mg of crabs immersed in 100% artificial sea water with high Mg (156 meq./l.) was 33.2 meq./l., which is significantly higher ($P < 0.01$) than the concentration of this ion for crabs which had been immersed in either 100% or 150% of natural sea water (20.0 and 27.1 meq./l., respectively). This is particularly interesting because as

TABLE IV

Ion concentrations in the blood of Pachygrapsus *immersed in small volumes of artificial sea water*

		50%			100%			150%		
		Mean	S.D.	No.	Mean	S.D.	No.	Mean	S.D.	No.
Na (meq./l.)	N	397	24	37	483	17.3	36	582	34	30
	0	410	30	15	493	9.6	14	562	40	15
	+	410	30	21	478	15.1	8			
	N/0	0.97			0.98			1.04		
	N/+	0.97			1.01					
K (meq./l.)	N	7.36	1.4	37	7.43	0.72	36	10.2	1.5	30
	0	5.78	1.0	15	9.19	0.82	14	8.62	1.4	15
	+	6.53	1.0	21	7.88	1.4	8			
	N/0	1.27*			0.81*			1.19*		
	N/+	1.13			0.94					
Ca (meq./l.)	N	34.8	7.9	24	29.6	5.9	44	36.4	4.8	30
	0	33.1	9.6	14	32.0	8.1	14	40.5	6.3	15
	+	29.7	6.4	16	38.1	8.4	17			
	N/0	1.05			0.93			0.90		
	N/+	1.17			0.78*					
Mg (meq./l.)	N	13.6	5.4	24	20.0	6.1	44	27.1	4.2	30
	0	13.2	5.1	14	11.7	5.7	14	16.7	7.5	15
	+	19.7	7.4	16	33.2	6.1	15			
	N/0	1.03			1.71*			1.62*		
	N/+	0.69*			0.60*					

N = crabs immersed in natural sea water.

0 = crabs immersed in Mg-free artificial sea water.

+ = crabs immersed in artificial sea water containing abnormally high Mg: in 50% sea water = 104 meq./l.; in 100% sea water = 156 meq./l.

* = significantly different from unity: $P < 0.01$.

indicated above, the urine Mg of crabs from this artificial medium is about equal in concentration to that of crabs from 100% natural sea water. It is apparent that the blood concentration of Mg is influenced by the influx of Mg from the medium, even though the Mg concentration in the urine is not directly affected.

Table IV also shows that blood Na is essentially unaltered by abnormal concentrations of Mg in the medium. It is interesting, however, that when immersed in 100% artificial sea water containing high Mg, the blood Na of *Pachygrapsus* remains normal, even though the concentration of this ion in the medium was reduced because of the high Mg. Blood Ca is unaltered in all conditions except when the crab is immersed in 100% sea water with high Mg. Here the blood Ca is significantly higher ($P < 0.01$) than for crabs from 100% natural sea water. There seems to be an interdependence between the regulation of Ca and Mg under these conditions. In Mg-free 50%, 100% and 150% sea water the blood K differs from the blood K of crabs from natural sea water. There is no consistent trend, but it can be suggested that there also is an interdependence between the regulation of Mg and K. Such a suggestion is supported by the above mentioned differences in urine K between animals from Mg-free sea water and those from natural sea water.

TABLE V

Apparent volume of distribution of sucrose in the blood space and water content for muscle in* Pachygrapsus *following immersion in different salinities*

Test medium (% sea water)	Mean volume of distribution (% body wt.)	S.D.	No.	Mean water content of muscle (% wet wt.)	S.D.	No.
50	15.4	1.39	10	76.58	1.77	18
100	18.7	3.45	11	75.00	1.40	25
150	26.7	5.34	14	71.70	1.71	34

* One minute after injection.

Table V demonstrates that the muscle tissue of *Pachygrapsus* gains water when the animal is immersed in 50% sea water and loses water when it is immersed in 150% sea water. That is, the muscle is permeable to water in both directions. This is particularly interesting inasmuch as the animal itself shows no significant weight changes during such treatments (Gross, 1957a). Table V also shows that the calculated apparent volume of distribution for sucrose one minute after injection into the blood space is smallest when the crab is in 50% sea water and largest when the crab is immersed in 150% sea water. This is interpreted to mean that the blood space volume of *Pachygrapsus* is altered when the animal is transferred from one salinity to another by the changing volume of the formed tissues. There may be objections to the use of only one concentration of sucrose for the injected dose (1 *M*), but the volume of the dose was no greater than 0.5 ml. and, if anything, would be expected to cause an increase in blood volume for crabs from dilute sea water and a reduced blood volume for crabs from concentrated sea water.

DISCUSSION

The diagram presented in Figure 1 suggests the course of events with respect to Mg and water fluxes when *Pachygrapsus* is immersed in different concentrations of sea water. Since the indicated Mg values are based on the crab's response to Mg-free media, it can be seen that the concentration of Mg in the urine of

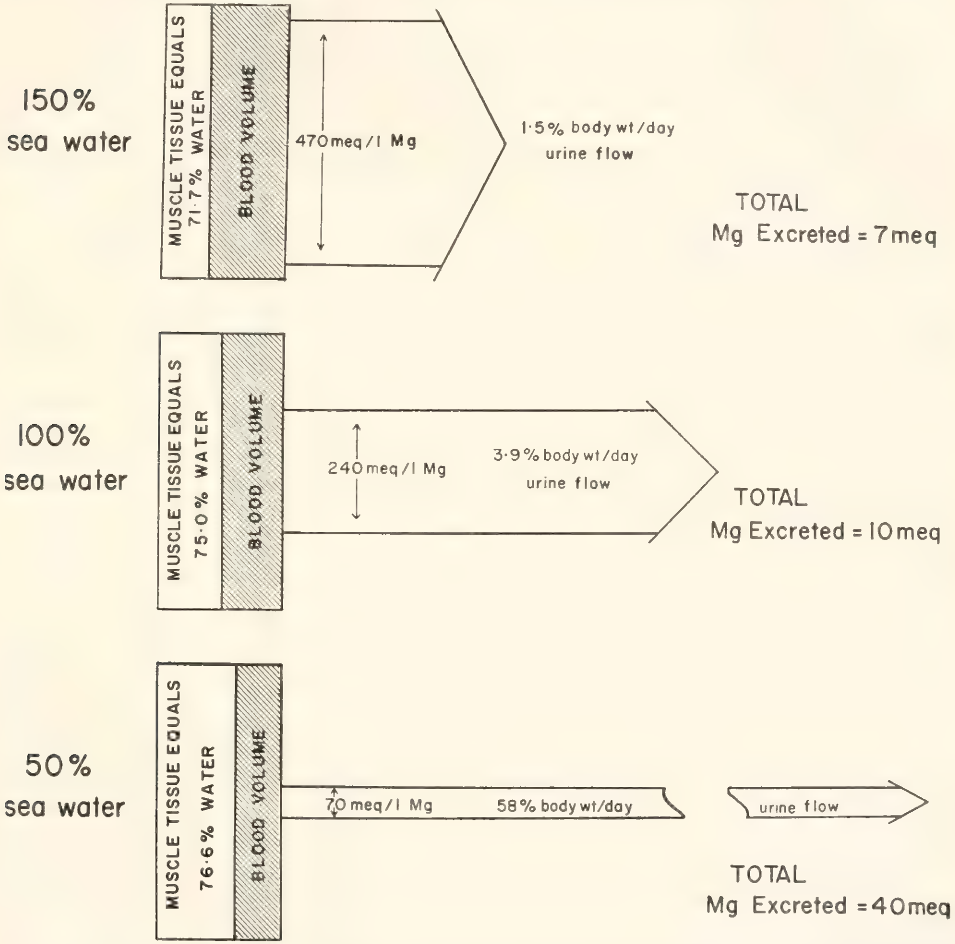
150% sea water
MUSCLE TISSUE EQUALS 71·7% WATER
BLOOD VOLUME
470 meq/l Mg
1·5% body wt/day urine flow
TOTAL Mg Excreted = 7meq
100% sea water
MUSCLE TISSUE EQUALS 75·0% WATER
BLOOD VOLUME
240 meq/l Mg
3·9% body wt/day urine flow
TOTAL Mg Excreted = 10meq
50% sea water
MUSCLE TISSUE EQUALS 76·6% WATER
BLOOD VOLUME
70 meq/l Mg
58% body wt/day
urine flow
TOTAL Mg Excreted = 40meq

FIGURE 1. Scheme of urine flow, water shifts and Mg effluxes in *Pachygrapsus* when exposed to different osmotic situations (indicated by the concentration of the external medium in % sea water on left side of diagram). Arrows which represent urine flow and Mg efflux are based on mean values for Mg concentrations in the urine and Mg losses to the medium when the crab is completely immersed in 50%, 100% and 150% Mg-free artificial sea water (see Table II). Length of arrow = volume of urine flow (% body weight/day); width = urine concentration of Mg (meq./l.); arrow area = relative amount of Mg excreted (meq.). Rectangles which are not drawn to scale represent crabs and illustrate the differences in blood volume (hatched area) in different osmotic situations, as suggested by the calculated volume of distribution for sucrose (Table V), and the volume of formed tissue (blank area), as suggested by the water content of muscle for crabs from different osmotic situations (Table V).

Pachygrapsus is directly related to the salinity of the external medium and not to the concentration of Mg in the medium or its influx into the animal. Likewise, it is shown that the efflux of Mg is inversely related to the concentration of this ion in the urine. (While the mean Mg loss in 150% was less than the mean Mg loss in 100% sea water, these values are not significantly different. However, the mean Mg loss in 50% sea water was more than four times the loss in the other two media.) Assuming that the antennary glands are the principal pathways for Mg efflux for all conditions, then small volumes of urine are produced in concentrated sea water and large volumes of urine are produced in dilute sea water. Figure 1 likewise shows that when *Pachygrapsus* is immersed in dilute sea water the water content of formed tissues is higher than when it is immersed in normal sea water, and conversely when the crab is immersed in concentrated sea water the water content of its formed tissues is less than when it is immersed in normal sea water. Such changes in water content effect volume alterations in the tissues at the expense of the blood space. As shown in Figure 1, then, the concentration of urine Mg is apparently determined by the water flux and is immediately independent of the concentration of this ion in the medium. Also, it is relatively independent of the Mg concentration in the blood. It will be observed (Table IV) that the blood Mg of crabs immersed in Mg-free sea water can be reduced below normal, yet the urine Mg (Table I) remains high (*e.g.* in 100% sea water). Again, in crabs immersed in 100% artificial sea water with high Mg the blood Mg becomes elevated above normal, but the urine Mg remains essentially the same as it is in crabs from 100% natural sea water. This response is contrary to the findings of Webb (1940), who demonstrated in *Carcinus* in a similar experiment that increases in the concentration of Mg in the external medium were reflected in the urine, but very little in the blood. Thus the mechanism of Mg regulation in *Pachygrapsus* may be fundamentally different from that of *Carcinus*. In the latter case the concentration of Mg in the urine increased even though the total salinity of the medium was normal; yet the blood Mg remained close to the concentration it attains in crabs from 100% natural sea water. As proposed by Webb (1940), excretion of Mg in *Carcinus* by way of the antennary glands does depend on the influx of this ion from the medium and consequently on the concentration of Mg in the blood. On the other hand, in *Pachygrapsus,* as shown above, the concentration of Mg in the urine is relatively independent of both the influx of this ion from the external medium and the concentration of Mg in the blood *per se*.

It seems, then, that in *Pachygrapsus* the concentration of urine Mg depends inversely upon the rate at which the antennary glands form urine or the rate at which the Mg-containing fluid is transported across the membranes of the antennary glands. This, in turn, depends on the magnitude and direction of water flux between the animal and its external medium.

The volume of urine flow calculated from the rates of Mg excretion seems reasonable for crabs immersed in 100% sea water. At least this value (3.9% body weight/day) agrees favorably with other values in the literature on other species, suggesting that this may be a valid method for estimating urine production. It is interesting that in a crab completely immersed in 150% sea water the calculated urine flow is 1.5% body weight/day, because there is a tendency for the crab to lose water to the hypertonic medium, yet there is no significant weight change even though fluid is being lost by way of the urine and probably by diffusion. The animal, therefore, must possess a mechanism for actively taking up water. Drinking is suggested as the principal method for replacing lost fluid. Burger (1957) has demonstrated drinking in *Homarus;* Green *et al.* (1959) have produced evidence that the gut takes part in the hypo-osmotic regulatory mechanism of *Uca.*

The excessive calculated urine flow for crabs completely immersed in 50% sea water (58% body weight/day) is difficult to interpret. It does not seem that such a rate could be sustained for long. This must mean that should this species inhabit water of such low salinities for prolonged periods, it must either alter its permeability or perhaps, being free to come out of the water, limit its period of immersion. The calculated rate of urine flow in the small volume of 50% sea water where the crab could rise out of the water was less than 10% body weight/day.

Again it becomes clear that the concentration alone of a given ion in the urine does not reveal the relative rate of excretion of that ion or, in the case of crabs, the relative quantitative role of the antennary glands in regulation of a particular substance. Table II reveals that the greatest mean loss of Mg was by crabs immersed in 50% sea water; yet these same crabs possessed the lowest concentrations of Mg in the urine. Conversely, the smallest mean loss of Mg was by the crabs immersed in 150% sea water which in turn had the highest Mg concentrations in the urine (Table II, Figure 1). In the case of crabs kept out of the water, the concentration of urine Mg was high (300 meq./l.), yet the amount excreted in three days could hardly be measured.

It is suggested from the lack of Mg loss when the crab is out of the water that *Pachygrapsus* must depend upon an uptake of water in order to excrete urine. This, then, is another physiological limitation binding this semi-terrestrial species to the sea. Gross (1955) discusses other characters which limit the terrestrial life of this crab.

It has long been recognized that when an aquatic animal enters a medium of a different salinity, it must undergo certain physiological adjustments which include the mechanisms of tolerance permitting adequate functioning of the cells and tissues despite changes in the salt concentration of the surrounding body fluids, or regulation which keeps those changes in blood concentrations at a minimum. Evidence has been produced by the present investigation that *Pachygrapsus* is capable of regulating the total water content of its body by expelling the excessive influx when in a hypotonic medium by a rapid flow of urine and, conversely, compensating in some way for the physical efflux of water when immersed in a hypertonic medium (perhaps by drinking). Table V, however, shows that the muscle tissue cannot regulate its volume, at least not during a three-day exposure to osmotic stress, the result being that the anatomy of the vascular system also becomes altered. It does not seem that the changes in the volume of the blood space suggested by data in Table V would not affect the efficiency of the animal.

It would seem, therefore, that should *Pachygrapsus* inhabit salinities which vary much from those of normal sea water for prolonged periods, it would be obliged to control the volume of its formed tissues so that a normal and efficient anatomy could be assured for the vascular system.

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We wish to thank Mr. John Bristow and Miss Mary Conlee for their able technical assistance. Also, we wish to express our gratitude to all those students who assisted in collecting the experimental animals.

SUMMARY

1. The concentration of Mg in the urine of *Pachygrapsus* is dictated by the salinity of the external medium and not by the Mg concentration in that medium or by the rate of Mg influx from the medium into the animal. Thus, during brief periods of immersion in 50%, 100% or 150% sea water the urine Mg concentration will reflect the salinity of the medium, irrespective of whether Mg is absent or in abnormally high concentrations.

2. The Na concentration in both blood and urine is not drastically altered by abnormal Mg levels in the external medium of any salinity tested.

3. After immersion in Mg-free 100% and 150% sea water the urine K of *Pachygrapsus* is higher than it is after immersion in the respective concentrations of natural sea water. Urine K is not influenced by the Mg concentration of 50% artificial sea water or by abnormally high Mg in 100% sea water. Blood K concentrations are affected by varying concentrations of Mg in the external medium of both dilute and concentrated salinities, but there is no definite trend.

4. The concentration of Ca in the urine of *Pachygrapsus* is unaffected by the Mg levels of all salinities tested. Blood Ca was not observed to be altered by abnormally high or low Mg levels in all media tested except in 100% artificial sea water with high Mg (156 meq./l.), where the blood Ca was significantly higher than for animals from 100% natural sea water.

5. While the concentration of urine Mg is not determined immediately by the influx of this ion into the animal, the blood Mg concentration is lowered when the crab is immersed in a Mg-free medium and raised when the medium Mg is abnormally high. The concentration of urine Mg is relatively independent of the levels of Mg in the blood.

6. *Pachygrapsus* excretes more Mg in 50% sea water than in 100% sea water and perhaps less in 150% sea water than in 100% sea water, even though the concentration of Mg in the urine is in the reverse order (*i.e.*, 150% > 100% > 50%).

7. Calculated rates of urine production for *Pachygrapsus* completely immersed in different salinities follow: in 150% sea water, 1.5% body weight/day; in 100% sea water, 3.9% body weight/day; in 50% sea water, 58% body weight/day. The observed rate for crabs immersed in 50% sea water is not believed to be sustained for long, as suggested by the high mortality rate. When removed from water, the volume of urine excreted by *Pachygrapsus* is insignificant.

8. The concentration of urine Mg in *Pachygrapsus* thus is inversely related to the rate at which urine is produced by the antennary glands and this is dependent on the magnitude and direction of the water flux, imposed by the physical gradient between the crab and its external medium.

9. The volume for muscle tissue in *Pachygrapsus* increases when the crab is transferred from normal sea water to dilute sea water and decreases when it is transferred to concentrated sea water. Such volume changes take place at the expense of the blood space.

10. It is suggested that alterations in the volume of the blood space caused by osmotic stress likely would reduce the efficiency of the vascular system which in turn would impose further ecological limitations on this species.

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NEUROMUSCULAR PHYSIOLOGY OF A SESSILE SCYPHOZOAN

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Interest in the coelenterate neuromuscular system has persisted for many years, and information gained by a variety of approaches. Pantin (1935a, 1935b, 1935c, 1935d) first introduced the use of controlled electrical stimuli and the observation of subsequent muscular contractions as a means of analysis of the properties of the conducting system in Actiniaria. Bullock (1943) has shown that the propagation of the swimming pulse in Scyphozoa differs in no fundamental way from excitation in anemones, although there are certain quantitative differences that result in the remarkable dissimilarity of behavior of the two groups of organisms. In anemones, contraction of the muscles is relatively rapid, but relaxation is slow, and because of neuromuscular facilitation each impulse after the first arriving at the muscle excites further contraction so that a typical "staircase" is recorded from the contraction of the sphincter muscle in *Calliactis*. In scyphozoan medusae the first impulse is effective in eliciting a response. Each succeeding impulse, if delivered within a well defined time limit, enhances the response for several contractions until a plateau is reached which may be maintained for long periods of time. After each contraction the muscle relaxes completely (or almost so) so that the typical kymograph record appears as in Figure 1, a recording of *Cyanea* bell contractions. This is the result of rapid contraction, rapid relaxation, the restoring force of the mesoglea, and an extraordinarily long absolute refractory period (0.7 second, according to Bullock, 1943) of the muscles of the medusa bell.

As indicated above, all critical work using controlled electrical stimulation has been confined to Anthozoa and free-swimming jellyfish. This raises the question of the relationship of response to mode of life. Are the phenomena described sharply divided along systematic lines, or are there some definite and demonstrable properties that may be associated with the sessile habit or the free-swimming habit? The Stauromedusae, about which very little is known, offer ideal material for such a study.

Except for a brief creeping larval phase, Stauromedusae are sessile throughout their existence, but there is no doubt of their affinities with the Scyphozoa. Do they retain the neuromuscular patterns of their nearest relatives, or has the sessile habit led to modifications (or retentions from the scyphistoma larva) that make the Stauromedusae functionally more closely associated with the anemones? Although Stauromedusae lack the ability to perform pulsating swimming motions, one might wonder if the system is capable of such contractions under artificial stimulation. Is the absence of a pacemaker the only essential factor here?

The comparative aspect of the problem would be ideally developed by studying:

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(a) the responses of anemones; (b) the responses of free-swimming Scyphozoa; (c) the responses of the scyphistoma larva of the Scyphozoa; and (d) the responses of Stauromedusae. A knowledge of all of these might enable one to assess the consequences of the sessile habit and give some insight into the evolution of their neuromuscular mechanisms and behavior. The present study deals only with the last of these.

MATERIALS AND METHODS

Haliclystus auricula (Rathke) was used because of its abundance near the Friday Harbor Laboratories of the University of Washington. Large individuals were collected, along with a portion of the blade of *Zostera* to which they are normally attached. The animals were maintained in the laboratory in running sea water at a temperature close to that of their natural environment (10–13° C.). Reactions were first explored by means of manually controlled mechanical stimuli and later by controlled condenser discharge shocks. Recordings were made with very light isotonic levers on a smoked drum.

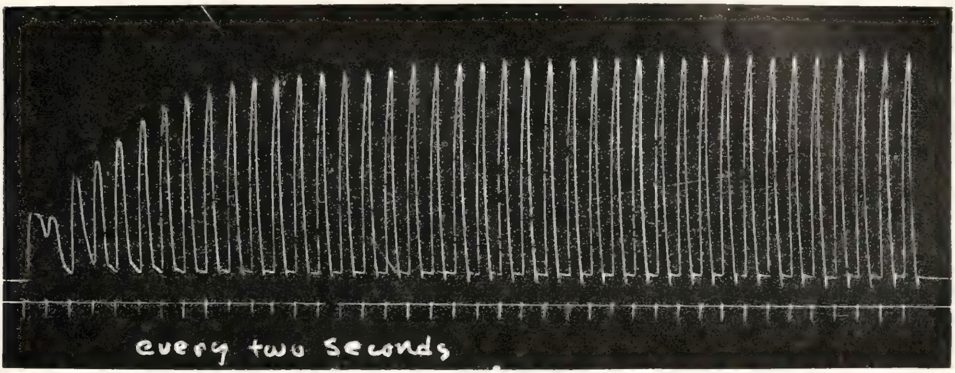

FIGURE 1. *Cyanea capillata.* Record of bell contractions. Electrical stimuli, 0.5 per second.

Electrical stimuli were delivered under sea water to the exumbrellar surface, usually at the base of the stalk or at the stalk-calyx junction, by means of silver electrodes insulated to the tip, with Ag-AgCl-sea water electrodes or non-polarizable calomel electrodes. A student "Electrodyne" stimulator, which delivers brief condenser discharge shocks, and for some experiments a Grass model S4B stimulator, were used. All experiments were made on fresh animals at temperatures of 11–13° C. At temperatures above 16–18° C. responses became very erratic, probably due to the rapid decay of facilitation (Hall and Pantin, 1937; Pantin and Vianna Dias, 1952). Mechanical stimuli were delivered with a clean, blunted glass rod or with a glass rod tipped with a short length of silver wire.

Anatomical studies to determine the details of the musculature were carried out by observation on entire living and preserved animals under reflected and transmitted light and between crossed polaroids. To confirm the results obtained from a study of entire animals, portions of several were sectioned and stained with picro-indigo-carmine following bulk staining in Grenacher's borax carmine, or with alum hematoxylin and eosin.

I would like to express my gratitude to the Director and Staff of the Friday Harbor Laboratories of the University of Washington for a grant which made residence at the Friday Harbor Laboratories possible for a period of seven months. My thanks are also due to the Department of Zoology, University of California, Berkeley, for support and encouragement over a period of several years. Dr. Ralph I. Smith and Dr. Cadet Hand have aided in many important ways, not the least of which was a critical reading of the manuscript. Their interest and criticism is deeply appreciated. Dr. T. H. Bullock has been kind enough to read the manuscript. His criticisms and suggestions have been very helpful and are gratefully acknowledged.

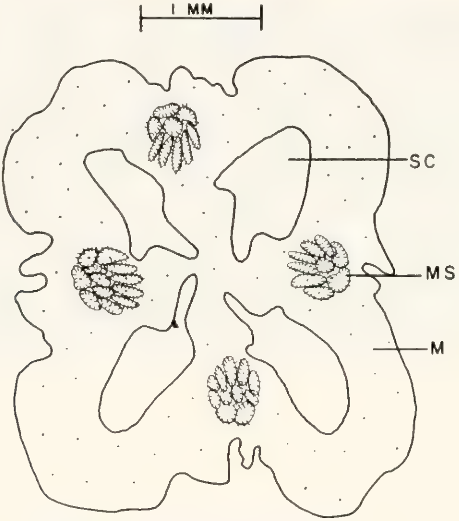

FIGURE 2. Cross-section through the stalk of *Haliclystus auricula*. SC, stalk canal; MS, stalk muscle; M, mesoglea.

RESULTS

Musculature of Haliclystus

It is possible to divide the muscles of *Haliclystus* into three categories associated with particular regions of the body. The stalk possesses four interradial muscular bundles (Fig. 2) embedded in the mesoglea which continue distally into the subumbrellar ectoderm as eight perradial sheets that fan out over the subumbrellar surface (Fig. 3). Proximally, the stalk muscles are inserted at the pedal disc over a rather wide area. There is also a marginal muscle (subumbrellar) which borders the margin of the calyx (Fig. 3) and is only partially embedded in the mesoglea. This latter muscle is interrupted at the arms and so consists of four perradial and four interradial segments. In addition, arm tentacles, gastric tentacles, manubrium, and anchors are provided with muscle fibers. The principal muscles involved in the reactions studied, however, are those of the stalk, subumbrella, and margin. With the possible exception of the stalk musculature, all the muscles of *Haliclystus* are confined to the subumbrellar surface, just as in free-swimming Scyphozoa. Thiel (1936) states that there are probably fine muscle fibrils distributed all over the body, although their existence has not been established, and no particular function has been ascribed to them. I have been unable to determine the presence of exumbrellar muscle fibers in *Haliclystus* and believe that all of the gross actions of the organism can be explained on the basis of interactions of the well-defined muscle fields and the mesoglea.

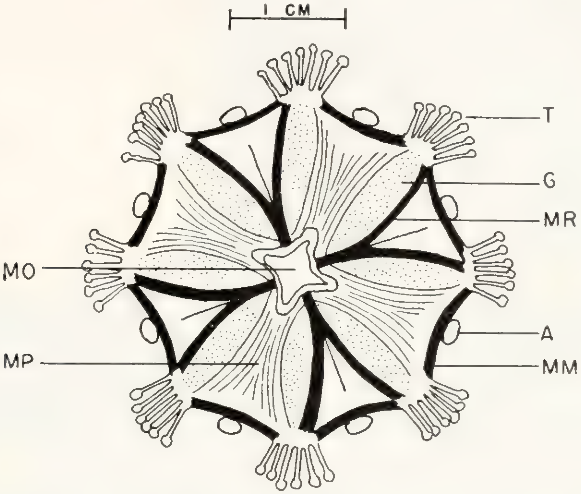

FIGURE 3. Diagram of *H. auricula* subumbrella to illustrate musculature. T, tentacle group; G, gonad; MR, radial muscle; A, anchor; MM, marginal muscle; MO, mouth; MP, perradial muscle.

Spontaneous activity

A considerable amount of apparently spontaneous activity may be observed in *Haliclystus* under both field and laboratory conditions. The arms are flicked in toward the mouth, the calyx is rotated, the stalk alone or the whole animal may contract without any noticeable external stimulation. No well-defined rhythm is apparent from visual observations, nor is any particular order of contracting parts consistently manifested.

Kymograph records of this activity were made on slow drums. There is no orderly progression of height of contraction as in free-swimming scyphozoan jellyfish. The contractions recorded are due primarily to the stalk muscles and do not show the contractions of individual arms. However, such recordings serve to illustrate the arrhythmic nature of spontaneous activity of unmolested animals (Fig. 4, A).

It was apparent from the outset that difficulty would be encountered in attempts to record responses to electrical stimuli in such an active animal. With this in mind, various parts of the body were isolated, to ascertain whether or not any structure or structures exercised a "pacemaker" control over any other part. Considering the systematic position of the animal, the most obvious place to look for such control was the umbrellar margin, which, in free-swimming scyphomedusae, bears the rhopalia, known to be the seat of pacemaker control of the swimming pulsations. In the majority of medusae (both Hydrozoa and Scyphozoa) removal of the bell margin results in a more or less complete paralysis of the swimming movements (Romanes, 1885; Bullock, 1943). No such pacemaker region has been demonstrated in anemones or in hydrozoan polyps, but in the case of anemones, the spontaneous activity is of such a slow nature as to allow investigation of neuromuscular responses with considerable confidence. In *Haliclystus* this activity occurs with sufficient frequency to be extremely troublesome.

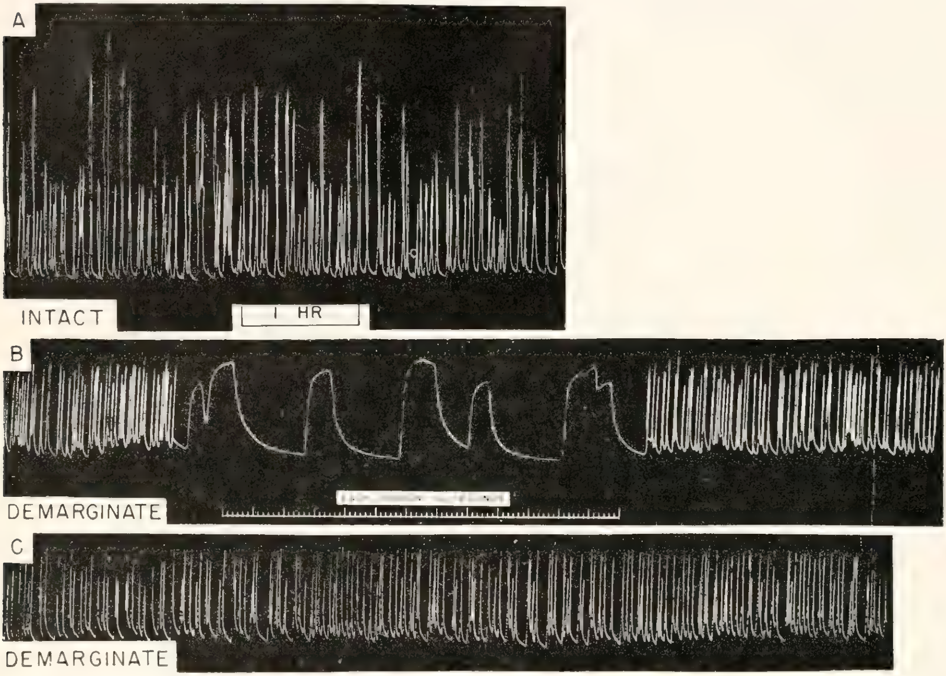

FIGURE 4. Records of spontaneous activity. A. Intact animal. B. Stalk only. Drum speed increased in central portion for 15 minutes. C. Stalk only. Hour marker applies to entire record except central part of B.

In order to determine whether or not "surgical paralysis" was possible, a large *Haliclystus* was set up in such a manner that stalk and calyx contractions could be recorded independently and simultaneously. Pins were placed at the stalk-calyx junction, fastening the animal to a wax-bottomed dish so that the two parts could be separated without disturbing the mechanical recording devices. Contractions of the intact animal were recorded for several hours, after which a sharp knife was drawn across the animal at the stalk-calyx junction, isolating each part from the other, and the recording continued for several more hours. Examination of the record shows that no difference can be observed in the nature of the contractions before and after separation. Further recordings were made of intact animals and isolated stalks, and no difference was noted (Fig. 4).

Removal of arm tips (tentacles), anchors, bell margin, and, as has been seen, the whole calyx, has no effect on the nature of spontaneous activity. From the above, it is apparent that there is no physiological centralization of the exciting system that manifests any pacemaker control over any other part. The mechanism of this type of activity has not been established directly, but may be inferred from evidence to be presented later.

Responses to mechanical stimulation

The stalk and exumbrella: Stimulation of the surface of the stalk and of the exumbrella in attached and unattached *Haliclystus* seldom elicited a response. Gentle stroking of the exumbrellar surface, stimulation by light touch, and fairly vigorous prodding almost invariably failed to call forth a response. If, however, firm pressure were maintained with the glass rod, *e.g.* on the edge of the pedal disc, a sudden shortening of the stalk and closure of the bell resulted. On several occasions the response was elicited only upon injury to the animal, and continued pressure did not cause a maintained calyx contraction. Shortly after the sudden closure the animal would relax, the calyx relaxing first, even if pressure sufficient to cause injury were maintained. This response could be obtained from any point on the exumbrellar surface and did not appear to be restricted to the pedal disc. The pedal disc does not appear to be any more sensitive than other areas, although the methods used would not give information on any slight gradient of sensitivity. After numerous attempts at mechanical stimulation of the exumbrella, it was concluded that the exumbrellar surface was very nearly insensitive to ordinary mechanical stimulation, responding only to very vigorous stimuli.

This is not surprising when one considers the conditions under which *Haliclystus* normally lives. The population from which experimental animals were taken is almost completely confined to rather dense beds of *Zostera marina*, with the animals usually attached to the blades in a pendant attitude. The *Zostera* is exposed only at the lowest tides, which means that for the greater part of the life-cycle of the animal it is hanging in the water among constantly moving blades of the plant, and is therefore subjected to a considerable amount of buffeting. The exumbrella would be exposed to repeated stimulation by blades of *Zostera*, debris in the surrounding water, and perhaps fairly frequent rubbing over the bottom. If these medusae were very sensitive to exumbrellar stimulation, they would be in the contracted state for a major part of their existence.

Subumbrellar surface and associated structures: The tentacles are extremely sensitive to weak mechanical stimulation. The slightest touch to a single tentacle usually brings about an arm-bending response. Sometimes only a few tentacles react, but more often there is a rapid bending of the whole arm toward the mouth. The arm tips at the base of the tentacles on the exumbrellar side are quite insensitive if the stimulus is applied outside the area of the tentacles, but are quite sensitive on the subumbrellar side.

The entire subumbrella is very sensitive to mechanical stimulation. If the stimulus is applied on the adradius, the arm in the position responds by bending toward the stimulated spot, and frequently the arm on either side also responds. If the stimulus is applied between the two arms (*i.e.*, in the inter- or perradius), the two adjacent arms respond by bending to the stimulated spot, and frequently, the next adjacent arm on either side also bends. In addition it was noted that the manubrium moved toward the stimulated area whenever the arm-bending response was obtained.

Repeated stimulation of a single spot on the subumbrella may either bring about a total calyx closure, or local insensitivity develops and the animal fails to respond. If a slight stroking movement covering several millimeters is made with a glass rod or silver wire, the total closure response results. It was noted on several occasions that repeated light touches in one spot would bring about total calyx closure in a fairly regular fashion. First the adjacent arms would bend toward the stimulated spot, then on the next stimulus the next adjacent pair, and so on until all eight arms were bent inwards. The exact relationship between stimulus and response in such cases was difficult to assess. If the stimulating object were not removed quickly, it would be caught by the tentacles and the stimulus of this contact would almost invariably cause total calyx closure. Quick removal of the glass rod, however, may have caused sufficient local disturbance to provide additional sources of stimuli, so that accurate appraisal of the stimulus-response relationship under these conditions seems impossible.

If the total calyx closure response were elicited by the stimulus of a linear stroke, stalk contraction and a characteristic rotation of the calyx usually occurred as well. The calyx rotation appeared simultaneously with the stalk contraction.

It was noted that any adequate mechanical stimulus delivered at the edge of the pedal disc always brought about a calyx closure reaction in addition to the stalk contraction, but that adequate calyx (subumbrellar) stimulation did not always elicit stalk contraction. This might be considered evidence of polarization in the conducting system, in the sense that it is "easier" to drive an impulse in one direction than in the other, but due to the methods of stimulation and the extreme difference in the intensity of "adequate" stimulation of the two areas concerned, it would seem unwarranted to accept this observation as evidence of polarity.

Conduction of excitation may be described as diffuse. A single touch elicits the arm-bending response in two to four arms, as well as a shift of the manubrium toward the stimulated point. The so-called "decremental" nature of conduction is also noted in the spread of contraction around the calyx from repeated or more vigorous stimulation. That conduction of excitation is rather slow is quite evident from observations made during mechanical stimulation. The arm nearest the site of stimulation always responds first, the other two following in order depending on whether the stimulated point is nearer one than the other. If they are equidistant they react simultaneously. The reaction is sufficiently slow so that the above is readily observed. Thus, the special properties of the nerve net as outlined by Pantin (1935a) are apparently fulfilled in *Haliclystus*.

Response to electrical stimulation

It has been indicated previously that the normal activity of *H. auricula* presents an unpredictable variable when one attempts to determine the responses of the organism to artificial stimulation. I found no convenient method of suppressing this activity. Further difficulty was encountered in determining the area to be stimulated. The demonstrated insensitivity of the exumbrellar surface offered the advantage that placement of the electrodes on it caused little disturbance, but this insensitivity may have been the result of absence of nervous elements in the exumbrellar ectoderm. If the electrodes were placed on the subumbrellar surface there was an immediate response to the tactile stimulation, and after a time local insensitivity developed.

The site finally selected was the exumbrella. Kassianow (1901) demonstrated what appears to be a nerve plexus in the exumbrellar ectoderm of Stauromedusae, and experiments to be described support this. Insensitivity to mechanical stimulation may be due to rapid sensory adaptation or to high sensory thresholds and paucity of mechano-receptors in the exumbrella. Neither of these possibilities has been directly demonstrated, but the responses suggest such explanations.

It was necessary to exercise caution in setting up the animals for recording. When possible, the animals were left attached to the *Zostera* on which they were collected and the plant pinned to the bottom of a wax- or clay-lined container. Small glass hooks were used to attach the animal to the recording lever. These were inserted through the bell margin beneath the marginal muscle at one side of an anchor. If the hooks were placed through the arm tips the animals remained unduly agitated for some time, presumably due to stimulation of the tentacles. Each preparation had to be observed carefully to eliminate the possibility of additional stimulation from the recording connections. Animals were not used until three to four hours after having been set up, to allow recovery from the effects of injury. Tests indicated that the responses did not differ appreciably after one-half hour from those after twenty-four hours following injury. All recordings were made from animals in standing sea water.

Response to single-impulse stimulation: As indicated previously, a single condenser-discharge shock results in a contraction of small amplitude in free-swimming Scyphozoa (Bullock, 1943). In the anemones that have been studied (Pantin, 1935a; Hall and Pantin, 1937; Pantin and Vianna Dias, 1952), single-shock stimulation does not result in a recorded response. To determine the response of *Haliclystus* to this type of stimulation, a large specimen was set up for recording and the electrodes applied to the stalk ectoderm.

As might be expected, the results were variable. Stimulation was being applied against a background of spontaneous activity. Contractions were recorded during the course of these experiments, but they were not always associated with the stimulus. Responses occurred before, after, and at stimulation in an apparently random manner. There was no consistent contraction associated with the delivery of the stimulus until high intensities were reached. Examination of preparations receiving such shocks revealed an injured area at the site of the electrodes, and these responses were interpreted as a result of multiple stimuli resulting from injury. Because of the fact that contractions occurred at random with this type of stimulation except at high voltages, it was concluded that *Haliclystus* does not characteristically respond to single-impulse exumbrellar stimulation.

The failure to respond to single-impulse stimulation also characterizes reflex closure of the sphincter of the anemone *Calliactis* (Pantin, 1935a) and the response of the longitudinal mesenteric muscles of *Metridium* (Hall and Pantin, 1937).

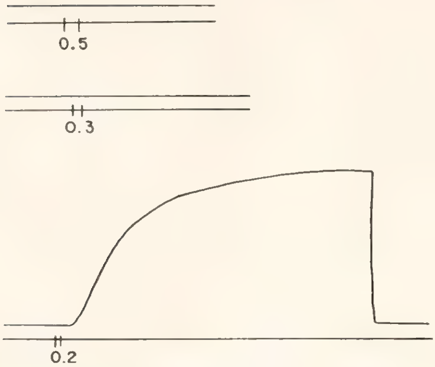

FIGURE 5. Tracings of records of stalk response to pairs of shocks. Upper trace of each pair, contraction record; lower trace, signal marker. Interval between shocks in seconds.

Following single-shock stimulation in *Haliclystus*, a twitching of the tentacles was often observed, as has been reported for *Calliactis* by Pantin (1935a), who considered it an indication that the impulse was being transmitted. In scyphozoan medusae there is a consistent measurable response to a single stimulus, although the magnitude of the response is very much less than that of which the preparation is capable (Bullock, 1943).

Response to pairs of stimuli: After having established the failure of *Haliclystus* to respond in a consistent manner to single-impulse stimulation, the response to pairs of stimuli was investigated. Pairs of shocks at a frequency of one in two seconds, and one, two, three, and five per second were delivered to the preparation. A standard five-minute rest period was observed between the delivery of each pair of stimuli, but because of spontaneous activity this interval was frequently extended. At least one minute of observed inactivity was allowed before the delivery of stimuli. A record of a typical experiment of this sort is shown in Figure 5. It will be noted that a response does not occur until the members of the pair of stimuli are separated by only 0.2 second, *i.e.*, at a frequency of five per second. Occasionally a response occurred at three per second, but the illustrated case is more typical.

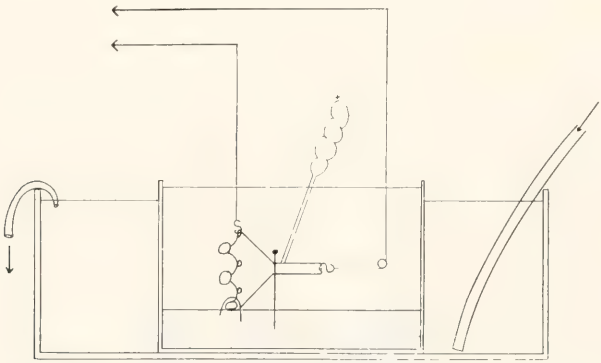

FIGURE 6. Diagram of experimental set-up for simultaneous stalk-calyx recordings.

During the course of the investigation described above, it was noted that total calyx closure regularly occurred at a lower frequency stimulation than stalk contraction. Because of the recording method, such calyx contractions were not recorded. Accordingly, animals were set up in such a way that independent and simultaneous records could be obtained of stalk and calyx contractions (Fig. 6). Stimuli were delivered on the exumbrellar surface at the stalk-calyx junction in the same manner as in the previous experiments. These were repeated on individuals without the encumbrance of the recording and holding devices, and in all cases the results corresponded to those shown in Figure 7. The calyx contracts weakly at a frequency of two per second, more strongly at three and five per second; at the latter interval the stalk contracts, as would be predicted from the previous determination. In contrast to the observed reactions to single stimuli, the responses to pairs of stimuli under the conditions described above are remarkably consistent.

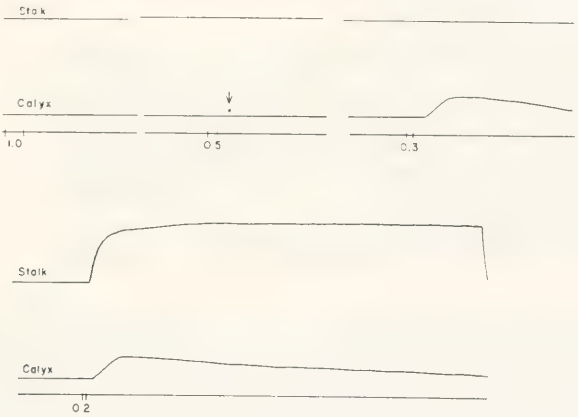

FIGURE 7. Tracings of records obtained as illustrated in Figure 6. Lower trace, signal marker. Interval between shocks in seconds. Unrecorded contraction observed at arrow.

Characteristic response frequencies for different parts of the responding system have been demonstrated in anemones by Pantin (1935b) and can be related to the function of the parts. In *Haliclystus* the calyx is the feeding organ of the animal and as such must be sensitive to very light mechanical disturbances if it is to fulfill its function.

Responses to trains of stimuli: The response to pairs of stimuli is essentially a total response. It is evident on observation of unmolested animals that natural responses are graded, *i.e.*, by no means all responses to naturally occurring stimuli involve all of the responding muscles to the full extent. It is also apparent that each of the three categories of muscles described earlier is capable of at least some independent action. The calyx is able to contract (as has been shown) without stalk contraction, and the stalk under certain conditions may show slow, partial, and frequently asymmetrical responses without the calyx being involved. This is apparent in the characteristic movements by which the expanded calyx is swept through the water, rotated, and inclined at various angles. The marginal muscle is also apparently capable of independent activity, as indicated by certain postures

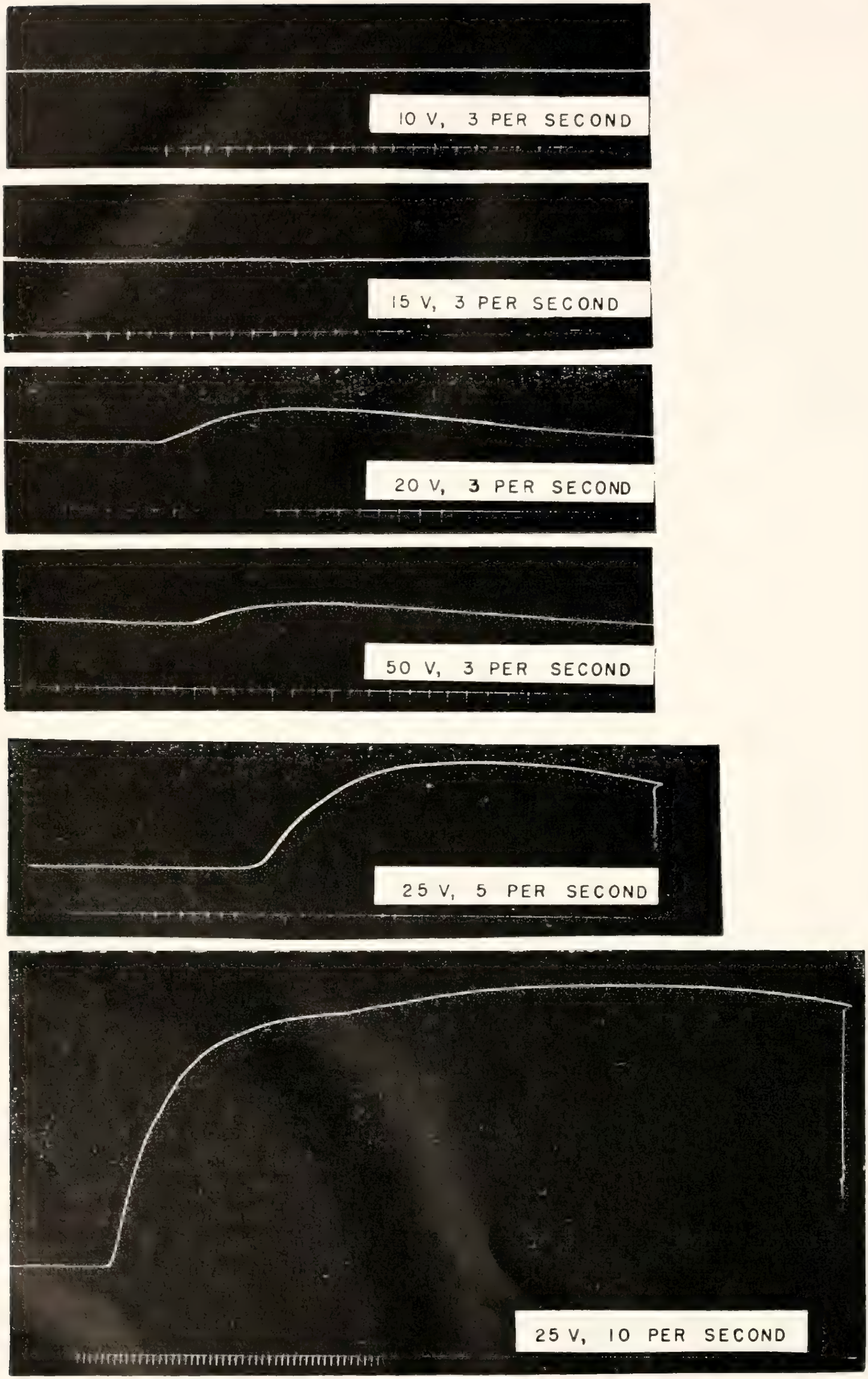

FIGURE 8. Records of stalk responses to various voltages and frequencies of electrical stimulation.

the animal assumes. In the expanded individual it is noted that the eight adradial arms are most frequently equidistantly arranged. At times, however, the arms are drawn together so that they are associated in four pairs, and such pairs may lie in the perradii or, more frequently, in the interradii. Examination of such animals reveals slight folding of the mesogiea over the marginal muscle between the pairs of arms and the corresponding radial muscles of the subumbrella. The intervening muscles show no sign of contraction.

In order to investigate the underlying mechanism of the graded response, the reactions to a variety of stimuli were examined. No information concerning the mechanism of the postural response has been obtained, but certain inferences concerning symmetrical graded responses may be made from reactions to trains of stimuli at different frequencies. Because of the spontaneous activity of the animals, it was impossible to interpret the results when long trains of stimuli at very low frequencies were administered. Any observed contraction could just as well be spontaneous as a reaction to the applied stimuli. At intermediate frequencies, however, *i.e.*, lower than that required for a response to a single pair, but higher than one shock every two seconds, a consistent pattern emerged.

Figure 8 illustrates a possible mechanism of graded response. It will be noted that there is a well-marked threshold and that increasing the intensity of the stimulation does not appreciably affect the height of contraction. At voltages just above threshold, however, increased frequency of stimulation has a marked effect on contraction amplitude.

A similar experiment was conducted on an animal set up for simultaneous stalk and calyx recording, as in Figure 6. The results shown in Figure 9 were recorded from the same animal in the space of less than one-half hour. It will be noted that there is no evidence of a well-marked "staircase" in any of these responses, as has been observed in the anemone *Calliactis* by Pantin (1935a), but that the contractions are essentially continuous. This may be interpreted as the result of the relationship between speed of contraction and decay of facilitation if factors similar to those in other coelenterates are operative. There is none of the machine-like precision in *Haliclystus* that is demonstrated by anemones and free-swimming Scyphozoa. This is probably because of the complicating factors of frequent spontaneous activity, speed of contraction, and the rapid decay of facilitation.

Responses to subumbrellar stimulation: The responses to electrical stimuli reported above are from stimulation of the exumbrellar surface, and are essentially only symmetrical responses. Asymmetrical responses are clearly possible, as indicated by mechanical stimulation and by observation of the undisturbed animal. Certain of these reactions may be examined by subumbrellar electrical stimulation despite the disadvantages pointed out earlier. Such responses have not been recorded because of the weakness of the muscles involved. In some cases, isolated calyces were used, while in others the observations were made on intact animals.

Upon placement of the electrodes there was an immediate and sometimes repeated response, either asymmetrical or total in nature. Electrodes were placed as lightly as possible, but the response ensued upon contact, however light. The animal was then left undisturbed for at least fifteen minutes.

Because of the extreme sensitivity of the subumbrella, precise analysis of the neuromuscular properties was very difficult. Single-impulse stimulation frequently elicited a localized response involving only one or two arms, as was observed following light mechanical stimulation. Single-impulse stimulation was never observed to cause a generalized response, but pairs of stimuli at 0.5- to 0.1-second intervals would cause a generalized contraction. Pairs at greater intervals would cause either two localized contractions coincident with stimulus delivery or would spread to the adjacent arms, depending on the interval. If several stimuli were delivered at a frequency of one per second, the contraction would spread in both directions around the calyx, bringing successive arms into contraction coincident with each successive stimulus. Those already contracted would contract again. Thus, the total calyx response could be elicited in a stepwise manner.

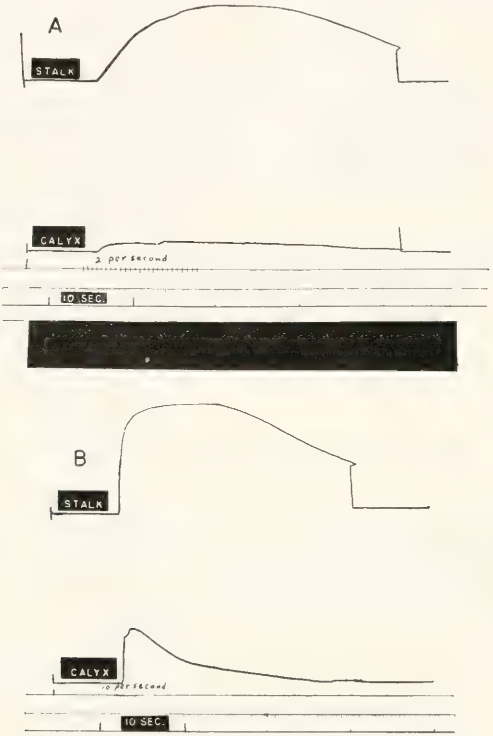

FIGURE 9. Simultaneous stalk-calyx contractions recorded as for Figure 7. Contraction records inked to increase contrast. A. Response to a train of shocks at 2/second. B. Response to a train of shocks at 10/second. Each preparation received 25 shocks.

A possible explanation of these results is that there is an immediate spread of the impulse to the neuromuscular junctions on the first stimulus and then neuromuscular facilitation governs the excitation of the muscle. A single pair of stimuli at a sufficiently short interval excites the whole system, but it takes several stimuli at lower frequencies to accomplish this. This is probably related to the rapid decay of facilitation, and suggests through-conducting tracts in the subumbrella which, on the basis of this evidence, might be syncytial. Evidence to be presented later, however, suggests that there is interneural facilitation as well.

Conduction speed in the "nerve net"

A number of attempts to measure speed of conduction in the system were carried out. Each determination was repeated several times before any consistency was obtained, and even then there was considerable variation. A strip of tissue was cut from the calyx, leaving one end of this tongue attached to the animal, which was then pinned to a wax-bottomed dish with the stalk projecting upwards. The stalk was connected to a recording lever in the usual way. A pair of stimuli was delivered at an appropriate interval distally on the tongue of tissue, and after five to ten minutes' rest, a similar pair was delivered at a measured distance proximal to the original site. The time from the second stimulus to contraction was carefully measured for each pair of stimuli, and the speed of conduction calculated from the difference in time and the distance between the stimulating electrodes. This was done on several animals, and the values obtained were 7–15 cm./sec. at 11–12° C., comparable to those of 10–20 cm./sec. obtained for *Calliactis* body wall by Pantin (1935b).

Other attempts were made by placing the electrodes at two different points on a line with the reacting muscles on the exumbrella in the intact animal. The results of such determinations varied so radically that they have not been included. Originally the assumption was made in these experiments that no conducting tissue crossed the mesoglea, and that the route of the impulse from stalk stimulation was up the stalk ectoderm, over the bell margin and down the subumbrella to the stalk muscles. The results suggested that there was transmission through the mesoglea at some point along the route.

The form of the "nerve net"

The most careful study of the nervous system of the Stauromedusae is that of Kassianow (1901). In a very thorough investigation using various staining techniques, including vital methylene blue, Kassianow studied the histology and cytology of the nervous system and associated structures in *Lucernaria* (= *Calvadosia*) *campanulata*, *Haliclystus octoradiatus* (= *H. auricula?*), and *Craterlophus tethys*. He describes and figures a diffuse exumbrellar plexus of bi- and tripolar ganglion cells. The experiments to be described largely confirm Kassianow's morphological findings.

Exumbrellar transmission of excitation: In order to test for the presence of exumbrellar nervous elements, several large specimens of *Haliclystus auricula* were selected and a circular incision was made through the stalk ectoderm at a level four to six millimeters above the pedal disc (Fig. 10, A). Following the operation the animals were given two to four hours in which to recover and were then stimulated on the stalk ectoderm aboral to the cut. Under these conditions a contraction could not be elicited.

Stimulus intensities up to 100 volts failed to cause excitation at frequencies at and above threshold for normal animals. Simply transferring the electrodes to the oral side of the cut resulted in excitation at normal frequencies and voltages. Animals were examined for muscle and endodermal continuity before and after stimulation. All animals showed continuity, although there was some damage to stalk canals in all specimens except one which gave the same results as the others.

The stalk of another individual was split from the base up toward the calyx and a cut made from the inside out toward the ectoderm, leaving only the ectoderm in continuity, and stimuli applied basal to the cut (Fig. 10, B). Under these conditions excitation occurred at normal voltages and frequencies. Subsequent examination revealed that a strip of ectoderm two to three millimeters wide was the only continuous tissue except for the very superficial mesoglea underlying the strip. This indicates that conduction of excitation is diffuse. If the conducting tissue is in the form of fibers, these are probably diffuse; but if conduction is a general property of the ectoderm or other superficial layers, one would also expect the results obtained. The latter possibility seems unlikely, but is not excluded by the evidence.

It might then be assumed that the normal path of exumbrellar transmission is over the exumbrellar surface to the bell margin, over the marginal edge to the subumbrellar muscles and to the stalk muscles. To test this assumption, the entire subumbrellar surface, including the oral region, was removed from an animal (Fig. 10, C). The ring muscle was left intact, as were the distal ends of the gonads, the arm tips, and tentacles. Under these conditions there is no ectodermal continuity from any point on the exumbrella to the stalk muscles, and since the radial muscles of the calyx have also been removed, transmission cannot take place through them. Stimuli were then delivered at the base of the stalk, and at normal voltages and frequencies a response of the stalk muscles was elicited. Such a preparation does not respond to a pair of stimuli at 0.2-second interval as does an intact animal, which may indicate that the normal pathway of excitation has been interrupted. Also, this preparation still had some septal tissue, which may have given continuity.

To insure no marginal continuity, another animal had the entire upper portion of the calyx removed as well as the subumbrellar tissue (Fig. 10, D). Under these conditions, a contraction could also be obtained at normal voltages and frequencies, although it took four shocks at five per second to elicit the response.

Still another animal was completely demarginated, the stalk split from the base up to within one millimeter of the cut edge, and stimuli applied at the base of one-half of the stalk (Fig. 10, E). This meant that if excitation were transmitted to the other half, it would be forced to traverse the mesoglea at some point in the non-stimulated half after having gone through the one-millimeter strip of ectoderm at the cut margin. The four groups of stalk muscles are separated by mesoglea at this level, and there is no opportunity for transmission through the split base of the stalk. Under these conditions, the non-stimulated half responds to shocks at normal voltages and frequencies. In this preparation there was some continuous endodermal tissue at the oral end, but the previous demonstration of the necessity of ectodermal continuity for transmission in the stalk reduces the significance of this. Furthermore, it does not affect the contention of mesogleal transmission, because neither the endoderm nor the ectoderm is in contact with the muscles.

Wietrzykowski (1912) clearly shows the development of the stalk muscles from the "cordon cellulaire de la taeniole" which has its point of proliferation in a subumbrellar position. This means that the direct innervation of the stalk musculature from the exumbrellar ectoderm is a result of conducting tissue arising in the exumbrellar ectoderm and becoming associated with the musculature, or of con-

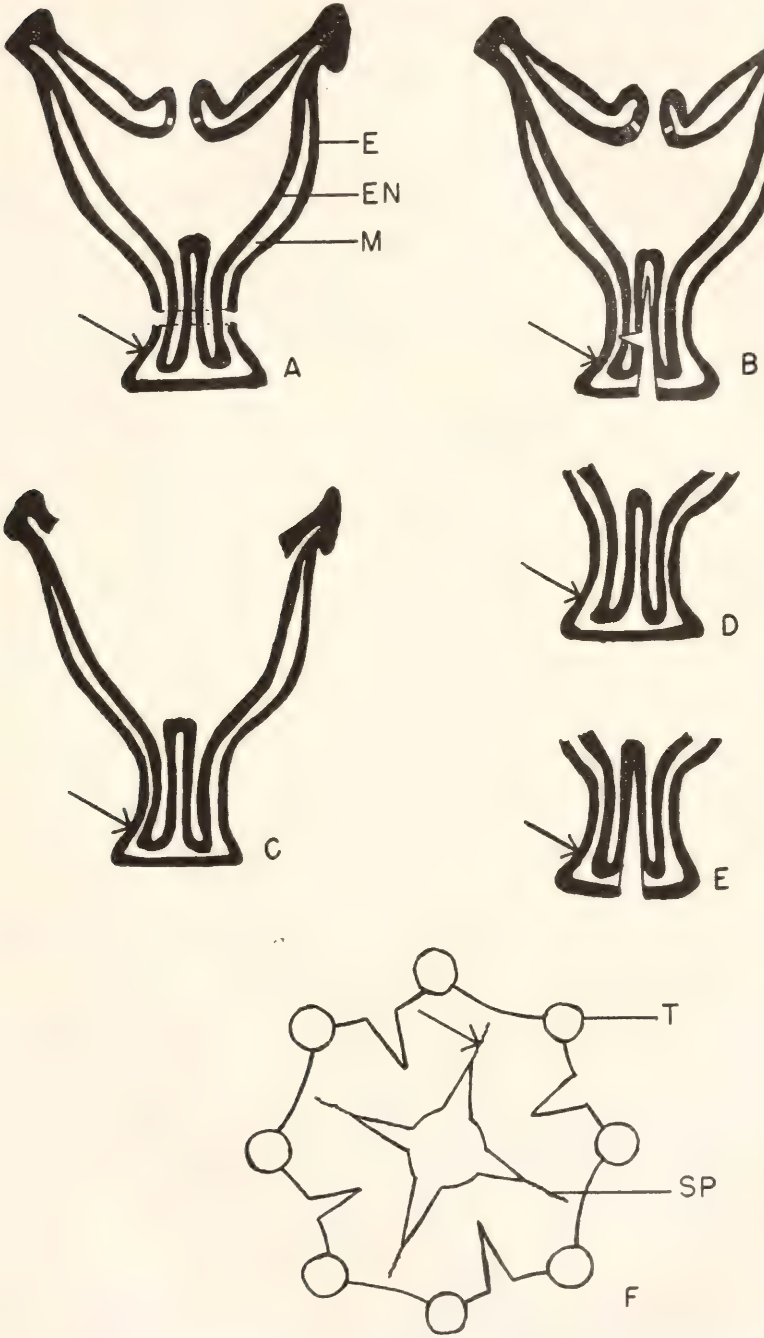

FIGURE 10. Diagrams to illustrate operations to ascertain the form of the conducting system. See text for explanation. E, ectoderm; EN, endoderm; M, mesoglea; SP, septum; T, tentacles. Position of stimulating electrodes indicated by arrows.

ducting tissue migrating out from the muscle to the ectoderm. Horridge (1956), however, has demonstrated a diffuse nerve net on the exumbrellar surface of the ephyra of *Aurellia aurita*, and it is possible that the exumbrellar conducting system of *H. auricula* is the homologue of the diffuse system in other scyphozoans.

Subumbrellar transmission of excitation: To ascertain the nature of subumbrellar conduction, the stalk and oral structures were removed from a specimen of *Haliclystus*. Four incomplete cuts were made from the margin to within a few millimeters of the cut inner surface between alternate pairs of arms, and similar cuts were made from the inside out toward the margin, alternating with the first cuts described (Fig. 10, F). This gave a ring preparation similar to those used by Romanes (1885) in which the impulse is forced to take a devious path to circle the bell. The electrodes were applied in the position indicated and trains of impulses were delivered to the preparation.

Under these conditions a train of impulses at normal voltage and at a frequency of five per second elicited a local contraction involving only the nearest two or four arms. At ten per second, the whole calyx could be brought into contraction in the following particular manner. First the arms nearest the electrodes respond, then those on each side in sequence and by pairs until all contract. There is a noticeable delay between the contractions of successive parts, as if junctions were being facilitated in turn. This preparation emphasizes the functional autonomy noticed in the intact animal. It also establishes the diffuseness of conduction and proves that no one pathway is essential for conduction around the bell. The expected pathway would be around the bell margin in the region of the marginal muscle, but the preparation described above shows that continuity of the muscle, and hence of any accompanying nerve tracts, is not essential to conduction. With arms and anchors removed, the preparation reacts in the same way as when these structures are present.

The above indicates that the arms are functionally associated into pairs. This functional pairing appears to depend on the integrity of the intervening marginal muscle and may indicate a normal through-conducting mechanism around the bell from arm to arm. Furthermore, if the stimulus is applied in the interradius, the contraction progresses by interradial pairs of arms. If applied in the perradius, the first reaction involves the adjacent arms only, or may involve the interradial *pair* on either side. This indicates that an interradial pair of arms, with its associated radial musculature (see description of musculature) and marginal muscle, has a degree of autonomy as a pair that separates it functionally from the other interradial pairs.

If the above results are examined in the light of stimulation of the intact calyx, the evidence for a through-conducting system is supported. That it is probably associated with the marginal muscle is indicated by the failure to respond to a pair of stimuli when this muscle is cut. It appears that under these conditions of mutilation, several junctions must be facilitated in order to bring about a generalized contraction.

Discussion

The results of this study enable one to construct a coherent picture of the normal behavior and responses of *Haliclystus auricula*. The pulsating contractions of the free-swimming Scyphozoa are lacking in *Haliclystus*. It has been demonstrated that there is no pacemaker present, and also that the requisite degree of specialization of the conducting system is lacking. Thus, the calyx of *Haliclystus* lacks the capability of responding in the repetitive fashion of its free-swimming relatives to repeated shocks at regular intervals. However, the available evidence indicates that a through-conducting system is normally operative in the subumbrella of the calyx. No evidence for higher conduction speed in that part of the animal, as compared to other parts, has been obtained. The evidence is primarily the different responses of experimentally incised and intact animals. The through-conducting system is brought into play only by relatively high frequency stimulation. At low frequencies the radial parts of the calyx exhibit considerable autonomy. In addition to this through-conducting system there also appears to be a diffuse "nerve net" which operates to correlate the subumbrellar structures, such as the manubrium, with the action of the principal food-gathering devices, the arms and the tentacles. There is no clear evidence of more than one nerve net as has been reported in *Aurellia* by Horridge (1956).

Inherent spontaneous activity may be interpreted in the following way: no part of the animal exercises control over any other part. From this we may assume that impulses are spontaneously generated in many, if not all, ganglion cells. Such impulses spread over the entire nerve net, but do not activate the muscles concerned unless two or more arrive at any one junction within an appropriate time interval. The nature of the spontaneous activity seems to indicate that each ganglion cell discharges at its own rate, each independently of the others. Activity may or may not be rhythmical. The graded and asymmetrical nature of the spontaneous activity, together with the lack of a regular pattern supports such an explanation. A pattern of endogenous activation of the muscles could lead to the observed sweeping, "seeking" motions of the unstimulated animal. Exogenous stimuli impinging upon the exumbrella must be of sufficient intensity to generate relatively high-frequency impulses if the protective closure-contraction response is to ensue, as indicated by the relatively high frequency of electrical stimulation required to bring about a response. The subumbrella, on the other hand, responds as a whole to lower frequency stimulation in a local fashion. Thus, if a food organism brushes very lightly over the subumbrellar surface a suitable reaction will be forthcoming in the arm-bending response to secure the prey. If the prey struggles, further impulses are generated, and the entire calyx responds to ensure capture and ingestion. The role of chemical stimuli in the predation process has not been investigated, but is no doubt of some importance—especially in the discharge of nematocysts and gland cell secretions.

If, as has been assumed, *Haliclystus* possesses a simple plexiform nerve net, the reactions reported in this study are subject to two possible interpretations. The high degree of autonomy noted in different regions of the subumbrellar structures is most easily explained on the basis of neuroneural facilitation at synapses between strategic areas of the net, or a hierarchy of thresholds of neuromuscular junctions to frequency and number of arriving impulses. Such a system, along with the endogenous activation of muscles, is admirably adapted to sweeping through the water in a "seeking" motion, and to catching prey on contact.

The exumbrellar nerve net shows a high degree of facilitation with rapid decay characteristics not noted in either anemones or free-swimming Scyphozoa, and this occurs at comparatively low temperatures (11–13° C.). The relationship of decay of facilitation (rapid) to contraction time (comparatively rapid) to relaxation time (relatively slow) is such that a fused contraction results at frequencies sufficiently high to elicit a contraction.

The subumbrellar nerve net exhibits the properties of through-conduction, neuromuscular facilitation and, probably, interneural facilitation. It responds locally to single-impulse stimulation, indicating a partial permanently facilitated state.

The properties of the neuromuscular system of *Haliclystus* do not appear to differ in any fundamental way from those noted in anemones (Pantin, 1935a, 1935d) and free-swimming Scyphozoa (Bullock, 1943). Modifications of the characteristics of the nerve net with regard to the time sequence of decay of facilitation and certain time characteristics of the muscles would appear to be sufficient to explain the differences in behavior noted in these animals. No new properties need be postulated to account for the reactions that do not fall in the range of variation already noted in the coelenterates.

Bearing in mind the presumed derivation of the Stauromedusae from free-swimming Scyphozoa, we may speculate upon the differences involved with the sessile habit. The most obvious of these is the loss of the pacemaker mechanism of swimming, and the loss of (or failure to develop) the specialized through-conducting mechanism that makes the swimming pulsations possible. Secondly, the stalk muscles are able to maintain a prolonged contraction not possible in free-swimming Scyphozoa. This is not as pronounced in the calyx musculature, which relaxes rather quickly even under sustained stimulation (see Figure 9). The properties of the mesoglea indicate that contraction is maintained against a mesogleal restoring force, hence the muscle must possess a refractory period of shorter duration than that found in free-swimming forms. Since sustained contraction differs in the stalk and calyx muscle systems, it is probable that each system has a muscle refractory period different from the other. Neither system is able to maintain a contraction for the length of time noted in anemones, so it may be reasoned that the physiological properties of both systems lie somewhere between those of free-swimming scyphozoans and those of anemones.

SUMMARY

1. Previous work on the neuromuscular systems of coelenterates has been confined to Anthozoa and free-swimming medusae, and certain differences in the response to electrical stimulation have been noted. It was thus of interest to investigate the responses of a sessile scyphozoan, *Haliclystus auricula* (Rathke), to determine the characteristics of the response mechanism.

2. Spontaneous activity of *H. auricula* is arrhythmic, and there is no demonstrable "pacemaker." The exumbrellar surface of the organism is relatively insensitive to mechanical stimuli, but the tentacles and subumbrellar surfaces are very sensitive. The conducting system possesses properties of a "nerve net." Conduction is slow, diffuse, and with an apparent decrement.

3. *H. auricula* does not respond to single-impulse electrical stimulation of the exumbrella. A pair of stimuli elicits stalk contraction at an interval of 0.2 second, and calyx contraction at 0.5 second. On the other hand, single-impulse stimulation of the subumbrella will elicit a response, but this response is local, involving only a part of the calyx near the position of the electrodes. A possible mechanism of the graded response is suggested.

4. Physiological evidence indicates that the conducting system is diffuse, with some evidence of a through-conducting pathway at the bell margin that operates at relatively high-frequency stimulation. Conduction speed in the subumbrellar nerve net is calculated at 7–15 cm. per second at 11–13° C.

5. The physiological properties of the neuromuscular system of *Haliclystus* appear to be intermediate between *Calliactis* and *Cyanea*. There is a demonstrable difference in the response of stalk and calyx which shows emphasis on sequential elicitation by mechanical stimulation of progressively greater intensity of tentacle, local marginal, essentially whole calyx, and finally symmetrical stalk involvement. The significance of this series of events in relation to the normal behavior of the animal is pointed out.

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SEASONAL CHANGES IN COLD–HARDINESS OF FUCUS VESICULOSUS

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Some marine algae, such as the fucoids, grow attached above mean low water and are, therefore, subjected at ebb tide to rather severe atmospheric conditions in some climates. In winter in the arctic, species of *Fucus* may be subjected to air temperatures down to − 40° C., yet do not seem to be at all injured (Scholander, Flagg, Hock and Irving, 1953; Kanwisher, 1957). The drying resistance of marine algae has been studied frequently in the past and it is apparent that those growing highest in the littoral zone are generally the most desiccation-resistant (Muenscher, 1915; Pringsheim, 1923; Isaac, 1935; Stocker and Holdheide, 1937; Biebl, 1939). The fact that they are desiccation-resistant suggests that they might also be fairly cold-resistant. This idea is borne out by findings of Kylin (1917) with *Fucus* and by those of Biebl (1958) with various marine algae.

In spite of this work, there appear to have been no seasonal studies of cold-hardiness of the common littoral marine algae. A number of such studies have been made with land plants (for example, Parker, 1955, 1959), and these have shown that there are wide fluctuations in cold-hardiness with the season in all woody plants of relatively cold climates. It therefore seemed of general biological interest to determine whether a marine plant such as *Fucus vesiculosus* L. would also go through such fluctuations.

METHODS

From June of 1958 to late May of 1959 *Fucus* plants were collected from convenient locations along the Long Island shore, near New Haven, as a preliminary study. Although there appeared to be a definite trend in hardiness from about − 20° C. in August to about − 60° C. in February, results were so irregular that it was suspected that some other environmental factor or factors besides air temperature were involved. It was therefore decided to repeat these experiments another year but to obtain the plants from a single location, both as regards the harbor and the position above low tide. It was found that hardiness varied as much as 15° C. in May between plants brought from about the mean low tide level and those brought from the highest fucoid zone, the higher ones being more resistant. This latter zone was used in the 1959–1960 experiments which are reported in this paper. It was decided that the stone jetty at Hammonasset beach, near Madison, Connecticut, was the best location, since it was accessible and the water relatively clean. An attempt was also made to obtain the plants as near to 2:00 P.M. as possible, in spite of variable tide levels for a particular time of day at different times of the year.

Fucus plants about eight inches long were plucked from the rock, put in stoppered jars of fresh sea water, and taken to the laboratory within 30 minutes. Fronds three inches long, including reproductive tips as well as vegetative ones, were cut from these plants, blotted on filter paper, and put in 500-ml. Dewar flasks in a damp condition. These were stoppered with a cork and cooled by an ethane compression system in a low temperature apparatus having a two-foot square compartment (Parker, 1959). The ethane, in turn, was cooled by a Freon-22 compressor system (Cincinnati Sub-Zero Products, Ohio). After being cooled at a rate of 4° C. per hour to one of the temperatures indicated by the blacked-in circles in the results (Fig. 1), flasks were removed to an ordinary refrigerator for three hours and then to room air to obtain a warming rate of about 8° C. per hour.

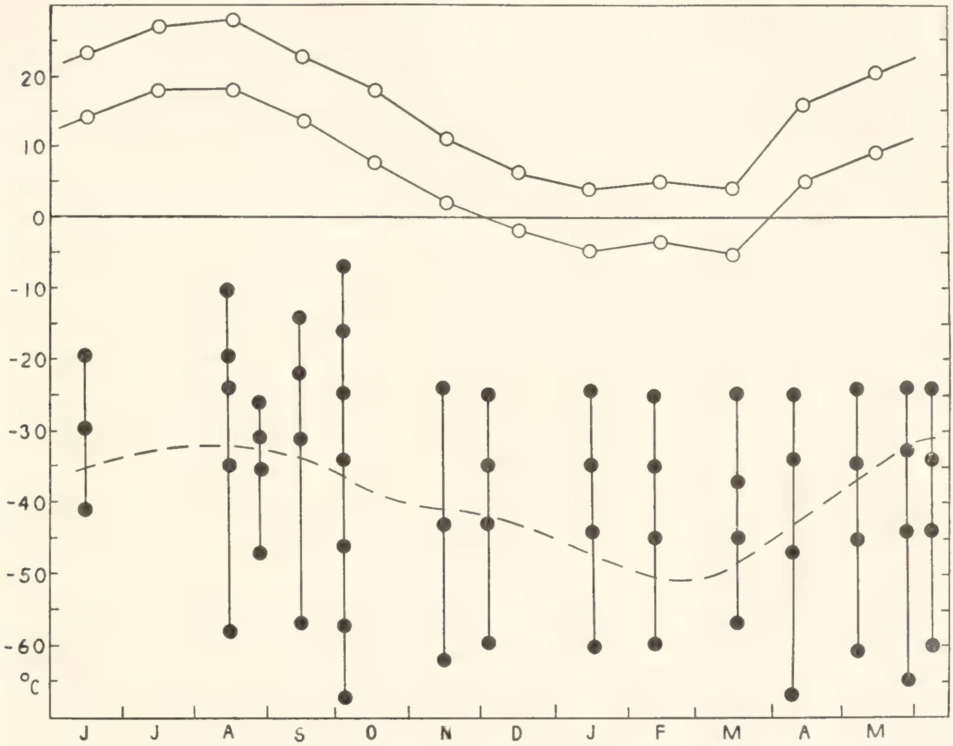

FIGURE 1. Upper curves represent maximum and minimum temperatures at the New Haven municipal airport, averaged for each month for the 1959–1960 season. Lower curve (dashed) represents the relative hardiness of *Fucus*, drawn rather freely as the approximate point at which 50% of the frond was alive (reducing tetrazolium chloride). Data are given in Table I. Blacked-in circles represent temperatures to which a group of plants were cooled on a particular day.

Determination of viability by ordinary means, for example by leaf color, is very difficult in these marine algae and may account for the lack of research in this field. But by means of the tetrazolium test, very clear and reliable results could be obtained. The fact that reduction of this compound by dehydrogenases to its red formazan derivative is a good indication of cellular viability has been previously discussed (Parker, 1953a, 1953b). One-inch-long cuttings from the tops of the cold-treated frond material were placed in 8-ml. test tubes and 6 ml. of a solution added, consisting of a 1 : 1 mixture of sea water and 0.6% 2,3,5-triphenyl tetrazolium chloride in tap water. Tubes were stoppered and placed in the dark for 4 to 18 hours at 23° C. Although some results can be observed in 4 hours, it was found best to wait 18 hours. After about 24 hours, bacterial action commonly interferes with results and clouds up the water with red precipitate. Reliability of the tetra-

TABLE I

Data shown in the lower part of Figure 1, together with effects of tetrazolium (TTC) test

June 15	Temp.*	−19	−29	−42				
	TTC test**	100	100	0				
Aug. 15	Temp.	−10	−19	−24	−36	−58		
	TTC test	100	100	100	40	0		
Aug. 29	Temp.	−26	−31	−36	−48			
	TTC test	100	50	40	5			
Sept. 10	Temp.	−14	−23	−32	−57			
	TTC test	100	100	40	0			
Oct. 5	Temp.	−8	−17	−25	−34	−46	−57	−67
	TTC test	100	100	100	100	5	0	0
Nov. 15	Temp.	−24	−43	−62				
	TTC test	100	5	0				
Dec. 4	Temp.	−25	−35	−42	−59			
	TTC test	100	100	10	5			
Jan. 15	Temp.	−24	−35	−43	−59			
	TTC test	100	100	80	10			
Feb. 12	Temp.	−25	−35	−44	−59			
	TTC test	100	100	80	15			
Mar. 18	Temp.	−25	−37	−44	−57			
	TTC test	100	100	60	50			
Apr. 10	Temp.	−25	−35	−47	−67			
	TTC test	95	100	10	5			
May 9	Temp.	−24	−35	−45	−61			
	TTC test	100	70	5	3			
May 31	Temp.	−24	−32	−44	−65			
	TTC test	100	5	0	0			
June 6	Temp.	−24	−34	−44	−60			
	TTC test	100	5	0	0			

* Temperature in degrees centigrade to which fronds were cooled.
** Frond surface in % showing positive tetrazolium test.

zolium test was also supported by the fact that *Fucus* failing to reduce the compound emitted a rotten odor in 48 hours while those reducing it did not.

RESULTS

Cold-hardiness was at a minimum during the summer at about − 30° C. (Fig. 1). Hardiness increased by October and by late winter reached about − 50° C. Since there were frequently borderline cases of damage, it was necessary to express results in terms of percentage tissue area reducing tetrazolium chloride (Table I). The dashed line in Figure 1 is drawn at approximately the point where there was 50% of the tissue not injured (reducing tetrazolium chloride). But even this system was somewhat complicated by the fact that in late summer, growing tips were more sensitive to cold than the rest of the plant, whereas in winter and early spring, tips appeared to be more hardy than the rest of the plant.

In spring, dehardening began to appear by mid-April when woody land plants were showing rapid dehardening. This was apparently related to the higher air temperatures of spring, beginning near the end of March. Surface water temperatures measured at the western end of Long Island Sound (Anon., 1947) and plotted as monthly means for a year showed little deviation from the air temperature data shown in Figure 1. It is therefore impossible to say whether air or water temperature is better related to changes in hardiness. There is, of course, no proof that seasonal changes in hardiness are not controlled endogenously or by some other environmental factor such as day-length.

SUMMARY

A seasonal study of the changes in cold-hardiness of *Fucus vesiculosus* L. was made over a two-year period. Plants in summer could withstand about − 30° C. (the lowest temperature at which 50% of the frond was still alive after treatment), whereas in January and February, plants could withstand − 45° C. to nearly − 60° C. Changes in hardiness with the season appeared to be related to air temperature variations, but also to mean monthly surface water temperatures plotted for a year. In May, *Fucus* taken from the low tide level was 15° C. more sensitive to the cold treatment than those from the highest level of the fucoid zone. Growing tips were the hardiest part of the plants in winter and early spring, but the least hardy in late summer.

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STUDIES ON MARINE BRYOZOA.

XIII. TWO NEW GENERA AND NEW SPECIES FROM ANTARCTICA

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The purpose of the present study is to describe two new genera, *Toretocheilum* and *Isoschizoporella*, and a new species, *T. absidatum*, from Marguerite Bay, Antarctica, and to elevate a previously known variety, *Schizoporella tumida* var. *tricuspis* Calvet 1909, to species rank: *Isoschizoporella tricuspis* (Calvet) 1909. The two species were collected for the Smithsonian Institution by the U. S. Navy's 1947–1948 Antarctic Expedition (hereafter referred to as USN) by Comdr. D. C. Nutt. The writer wishes to express her very grateful appreciation to the Smithsonian, U .S. National Museum, for the loan of these specimens and to the National Science Foundation for grants so generously supporting this and related researches.

Both species belong to the family Hippoporinidae as redefined by Osburn (1952, pp. 316 and 343). He limited the family to include those schizoporellid species which have a pleurocyst or olocyst type of frontal wall with marginal areolar pores (areolae), avicularia, usually strong cardelles, orifice and operculum constricted at sides, in some species at least.

GENUS *TORETOCHEILUM*, NEW GENUS

Diagnosis. Colony encrusting. Zooecia entirely adherent. Dietellae present (Fig. 9). Zooecial front wall convex, an areolate and moderately costate pleurocyst. Frontal not otherwise porous. Large pointed adventitious avicularia on front, over areolar pores (Fig. 1). Globose ovicell with areolae around border in ectooecium or where ectooecium and entooecium meet (Figs. 2, 3, 5). Ovicell partly immersed in next distal zooecium (Fig. 5). Zooecial operculum does not close ovicell aperture. Low spine-bearing peristome surrounds zooecial orifice (Figs. 1, 6, 10). Orifice suborbicular, laterally and distally bounded by a C-shaped vestibular arch which supports the operculum (Figs. 6, 7). Vestibular arch ends proximally on each side in thick ledge-like cardelle (Figs. 8, 10). Between the cardelles is a gap or inner sinus closed by the tab of the operculum (Fig. 8). In front of this, externally, the low peristome rises to form two curved cusps which encircle the rounded median peristomial sinus (Fig. 10).

Name derivation. The genus was named *Toretocheilum*, meaning "pierced lip," because of the sinus-pierced proximal peristome which forms the lower "lip" of the orifice. It is of Greek derivation (see Brown's lexicon) from *toretos*, bored or pierced, and *cheilos* (neuter noun), lip or rim.

Type species: Toretocheilum absidatum, n. sp.

Type locality: Marguerite Bay, Antarctica, Sta. 240.

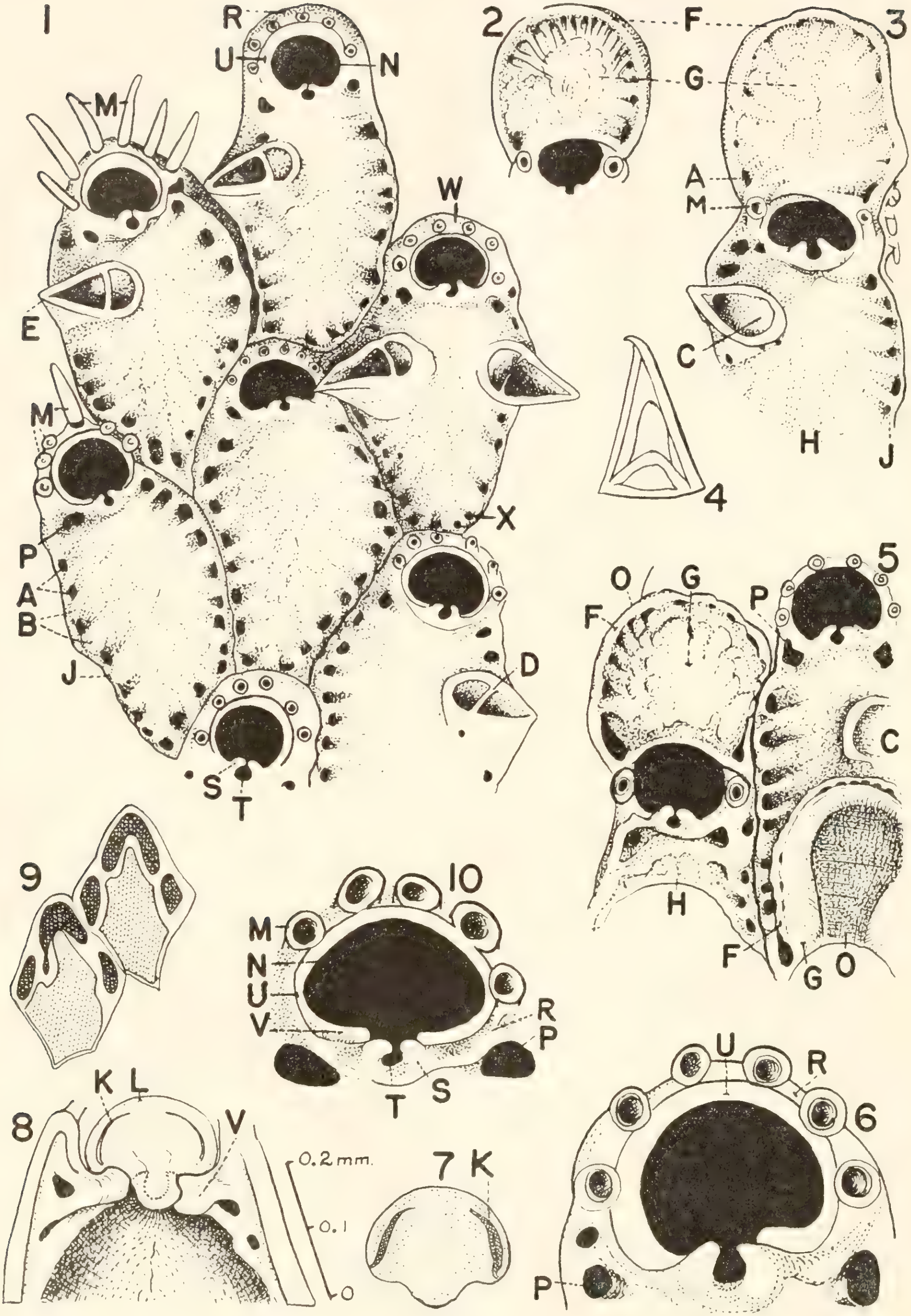
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PLATE I.

Remarks. The new species *absidatum* presented a problem in identification and relationships. It had features strongly allying it to both genus *Chiastosella* and genus *Stephanosella.*

Chiastosella is known only from the southern hemisphere and is represented by both fossil and Recent species. *Stephanosella* has a wider distribution.

Chiastosella is classified in different families by different authors. Stach (1937) and Brown (1952) allocate it to the family Schizoporellidae, while Bassler (1953) allocates it to the family Hippoporinidae, which was once a subfamily of Schizoporellidae but which is now a separate family. Bassler (1953) and Osburn (1952)

Torctocheilum absidatum, new species

LIST OF ABBREVIATIONS USED ON THE PLATES

A	Areolae or areolar pores	N	Orifice of zooecium
B	Areolar costae	O	Ovicell
C	Avicularial chamber	P	Paraoral areolar pores
D	Avicularial pivot	R	Peristome
E	Avicularium	S	Peristomial cusps
F	Ectooecium of ovicell	T	Peristomial sinus
G	Entooecium of ovicell	U	Vestibular arch or collar
H	Frontal wall	V	Vestibular ledge or cardelles
J	Mural rim	W	Distal part of zooecium
K	Opercular sclerite	X	Proximal part of zooecium
L	Operculum	Y	Distal wall
M	Spines or spine bases	Z	Lateral wall

PLATE I

All figures on this plate are of *Torctocheilum absidatum*, new genus and new species, and are drawn with the aid of a camera lucida.

FIGURE 1. Seven calcined non-ovicelled zooecia, some with one or two avicularia, some without any.

FIGURE 2. An ovicell drawn directly from the rock under low power (dissecting microscope). Above the two spine bases are the spaces ("pores") between ectooecium and entooecium.

FIGURE 3. A calcined ovicelled zooecium with an avicularial chamber in which the underlying areolar pore is visible. The floor of the avicularial chamber is the frontal wall of the zooecium.

FIGURE 4. Mandible of an avicularium.

FIGURE 5. Two young calcined zooecia, the one at left ovicelled, the one at right non-ovicelled. At lower right, imbedded in the proximal front wall of the latter, is a damaged ovicell which belongs to a zooecium below those shown. The front wall of the damaged ovicell is broken off, exposing the inner wall of the ovicell. The ovicell rim shown over the orifice later becomes reduced as calcification proceeds.

FIGURE 6. External view of orifice of a non-ovicelled zoid. One cardelle of vestibular arch is hidden by a peristomial cusp.

FIGURE 7. Operculum with curved sclerites for muscle attachment. The median tab below fits the space between the cardelles and the back of the cusps.

FIGURE 8. Interior of the frontal wall, operculum and half the orifice. The thick ends (V, ledges or cardelles) of the vestibular arch hold the operculum in place. Through the opercular tab can be seen the peristomial cusps and peristomial sinus. These peristomial structures are external to the operculum. Drawn to the 0.2 mm.-long scale at right.

FIGURE 9. The attached back or basal wall of two zooecia, showing the three distal darkly shaded dietellae of each.

FIGURE 10. Zooecial orifice tipped forward to show the exact relation of the C-shaped vestibular arch and vestibular sinus to the peristomial cusps and peristomial sinus.

place *Stephanosella* in the family Hippoporinidae. The difference between the two families is based on the nature of the orifice and the frontal wall.

The new USN species *absidatum* is closer to *Chiastosella daedala* (MacGillivray) 1887, type species of *Chiastosella* Canu and Bassler 1934 (see Bassler, 1934, p. 407) as regards the distinctive orifice, peristome, spines, and avicularia but differs in the type of frontal wall and ovicell porosity and sculpturing. In the latter features (frontal wall and ovicell) *absidatum* is closer to *Stephanosella* Canu and Bassler 1917, but the orifices are quite different.

Chiastosella at present contains a diversity of species (*cf.* Stach, 1937; D. A. Brown, 1952, 1954) that could split the genus between two families (Osburn's limited families Hippoporinidae and Schizoporellidae). Therefore, it was thought more sensible to erect a new genus *Toretocheilum* for the dissident and complicating USN *absidatum* than to further diversify the genus *Chiastosella* by including a new species of such divergence from the type *C. daedala.*

Diagnosis. Colony encrusting, well calcified. Zooecia convex and approximately hexagonal. Frontal a pleurocyst with one row of areolae, the paraoral pair often more pronounced. Areolar grooves and costae prominent. None, one or two large transverse, pointed avicularia over areolar pores on mid-frontal corners. Five to 7 stout oral spines on peristome. Orifice suborbicular, lined by a C-shaped vestibular arch whose ends form prominent blunt cardelles proximally. Proximal border of orifice straight to concave, with round, median sinus inserted between or flanked by two arched cusps. Ovicell with row of areolae where ectooecium and entooecium meet peripherally, but otherwise non-porous. Ovicell has faint proximal rim, bordered by two oral spines. Three or more large basal dietellae.

Name derivation. The species *T. absidatum* was named for the arched proximal peristomial cusps which outline the median orificial sinus, and also for the remainder of the arched orifice. The trivial name is derived from the Latin *apsis* (*absis*), arch; *absidatus,* arched, vaulted. (*Cf.* Brown's lexicon.)

Measurements. Given below are minimum, maximum and the average of a number of readings, usually 10, in millimeters. L is for length, W for width, D for diameter.

0.965–1.267	(1.081)	L	zooecia
0.432–0.763	(0.634)	W	zooecia
0.576–0.605	(0.586)	L	ovicell, 4 readings
0.547–0.576	(0.566)	W	ovicell, 4 readings
0.202–0.245	(0.217)	L	orifice, including sinus
0.216–0.245	(0.230)	W	orifice
0.014–0.058	(0.033)	D	sinus
0.259–0.331	(0.297)	L	avicularium
0.130–0.173	(0.151)	W	avicularium
0.216–0.346	(0.250)	L	oral spines, 6 readings
0.029–0.058	(0.045)	D	oral spines
0.163–0.202	(0.182)	L	mandible, 4 readings
0.124–0.130	(0.128)	W	mandible, 4 readings

Colony. Four small patches of colony were found encrusting a thick flat hand-sized rock from Marguerite Bay. Pieces had to be scraped or burned off for study because they were on the sides of the rock in areas that could not be maneuvered for study under the compound microscope.

Zooecia. Zooecia are box-like, their fronts 4- to 6-sided. The side walls are vertical, low and about the same height all around. Three or so large dietellae outline the distal half of the basal wall (Fig. 9).

The frontal wall is very convex, mound-like, with thin, raised mural rim. A row of deeply sunk oval areolae outlines it. Grooves radiate centerward from the areolae. Short costal ridges separate them.

The frontal wall consists of a translucent calcareous olocyst fast overgrown by an opaque granular or roughened pleurocyst. The central part of the pleurocyst is non-porous except for an occasional pore puncturing the avicularial chamber near its base. The areolar pores are not large but the grooves and ridges make them more conspicuous than would otherwise be the case. The two areolae nearest the orifice corners are often slightly larger than the others (Figs. 5, 6). In young, less heavily calcified zooecia the grooves leading from these two paraorificial areolae pass just proximal to the orifice, leaving the orifice elevated slightly above the immediate frontal wall (Fig. 5).

Avicularia. Avicularia are all of one type and of approximately the same size. Usually one, sometimes two, large pointed avicularia occur on some zooecia. Other zooecia may be without any. If two avicularia are present on one zoid they are placed bilaterally, opposite each other. Avicularia are located at the corners over areolar pores, midway up the side of the front wall. They are transversely oriented with mandible pointing out. Sometimes they slant a bit obliquely (Figs. 1, 3).

Each avicularium is mounted on a prominent avicularial chamber which tips the avicularial beak to a slightly upward-directed or oblique position. The avicularial chamber is large at the base and covers one or two areolae. As is the custom in calcified species with large adventitious avicularial chambers, there is an occasional small pore perforating the chamber wall near its base, probably for hydrostatic reasons.

The avicularial back area is hemispherical. The beak is pointed. Separating them is a straight pivot bar on which the base of the triangular mandible rests. The mandible is a tall narrow triangle, with curved tip and a lucida near its base (Fig. 4).

Orifice. The unusual orifice of *T. absidatum* made it difficult to classify because either few species have this type or else if they have it the illustrations generally do not show the orifice in very great detail.

The orifice is tipped forward a bit distally so that it seems horizontal. In front of it the frontal wall soon rises like a mound. The orifice is placed at the very extremity of the frontal wall.

It is lined by a C-shaped inner collar, the vestibular arch, whose ends curve around proximally to form the cardelles (Figs. 8, 10). The cardelles are sturdy, blunt, smooth usually and separated from each other by a median gap approximately one-third the width of the orifice.

In front of the gap is the rounded spout-like sinus whose walls are formed by the two curving cusps which arise from the proximal wall of the peristome (Figs. 6, 8, 10). The sinus and gap form an inward slanting channel. In young zooecia the orifice with the frontal peristomial tabs and sinus is set off from the zooecial

Isoschizoporella tricuspis (Calvet) 1909

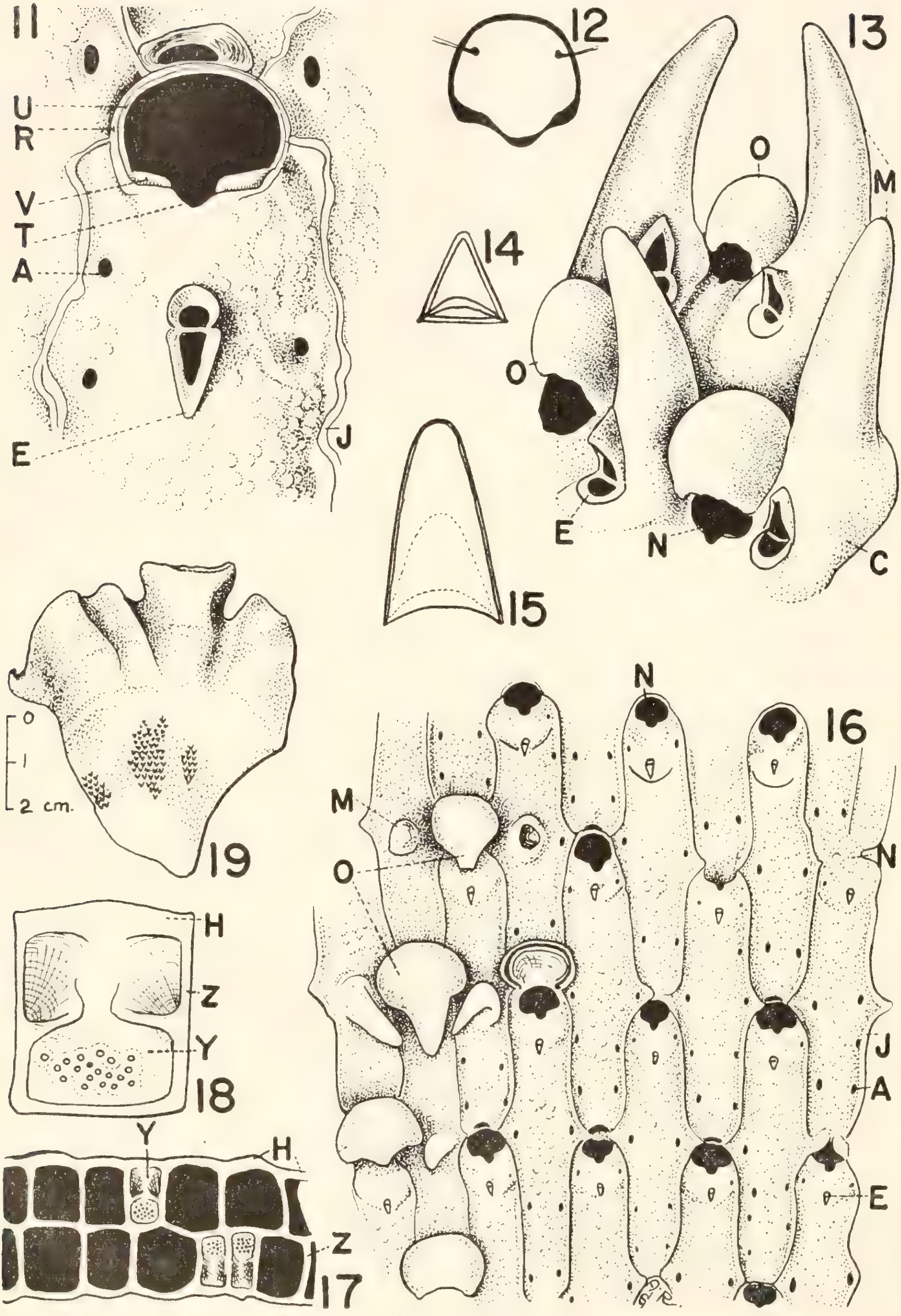
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Plate II.

front by a crease or groove that extends between the two paraorificial areolae (Fig. 5) but this later is obliterated. In *C. daedala* the cusps are transverse. In *T. absidatum* they are more vertical or obliquely arched.

Operculum. The operculum is rather delicate but reinforced by a long curved chitinous sclerite or reinforcement at each side (Figs. 7, 8). The sclerite is for muscle attachment. The extent or length of the sclerite depends on the degree of chitinization of the operculum. The operculum is shaped to fit the distal semicircular anter of the orifice and the intercardellar gap rather than the more external sinus.

Spines. Non-ovicelled zoids have 5 to 7 peristomial spines around the hemispherical part of the orifice. On ovicelled zoids the distal part of the orifice is not visible, so whether spine bases are present distally cannot at present be determined, but there is a thick spine at each side of the orifice just proximal to the side of the ovicell (Figs. 2, 5).

The spines are coarse and jointed at the thick base. The bases of the proximal pair are a bit sturdier or bigger on some zoids.

Ovicells. In general appearance the *Toretocheilum absidatum* ovicells resemble those of the genus *Stephanosella* and of Stach's *Chaestosella gabrieli* and Brown's *C. enigma.*

The *T. absidatum* ovicell is deeply immersed in the frontal of the next distal zoid. Also, it hides the distal part of the orifice of its own zoid. Its very own aperture cannot be seen from the front because the ovicell overhangs so. The peristome does not encroach upon the ovicell.

The ovicell has two calcareous layers, the ectooecium and the entooecium. Brown's (1952, p. 36) interpretation of these two layers is here followed. The layers are separated by a very narrow space. The entooecium is globose, rough-

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PLATE II

All figures on this plate are of *Isoschizoporella tricuspis* (Calvet) 1909. All except Figures 14 and 19 are drawn with a camera lucida. Figure 13 is from Sta. 226 material, the rest are from Sta. 234 specimens.

FIGURE 11. Detail of distal third of zooecium, showing well calcified beaded frontal wall and a characteristic uncalcified space above the orifice. The avicularium is prominent enough but the avicularial chamber is immersed and the heavy calcification makes it inconspicuous.

FIGURE 12. Operculum. Tendon fibers are attached to the two distal muscle dots.

FIGURE 13. Three ovicells flanked by aviculiferous spines.

FIGURE 14. Diagram of a suboral avicularial mandible.

FIGURE 15. Mandible of a spinal avicularium.

FIGURE 16. Portion of a colony showing both ovicelled and non-ovicelled zooecia. Some zooecia have the large frontal spines. One of the spines and one ovicell are broken, in the fourth row from the left. In the row at extreme right the two lower zooecia show degrees of occlusion of orifice by secondary calcification. The orifice of the upper one is completely calcified and overgrown. That of the lower right is partly so. The upper zooecium has given rise to two new rows of zooecia.

FIGURE 17. Cross-section through 15 zooecia of a bilaminate colony. Zooecial cavities are in black, zooecial walls are in white. The two layers of zooecia are back-to-back. The three filled in compartments are the end walls (*cf.* Fig. 18).

FIGURE 18. End or distal wall of a zooecium, showing the interzoidal communication pores or sieve plate in bottom half and the pattern or lines of calcification in the upper half.

FIGURE 19. Piece of a bilaminate blade or frond of a colony, drawn to the 2 cm. scale at left. The three darker patches on the lower half are spined and ovicelled areas.

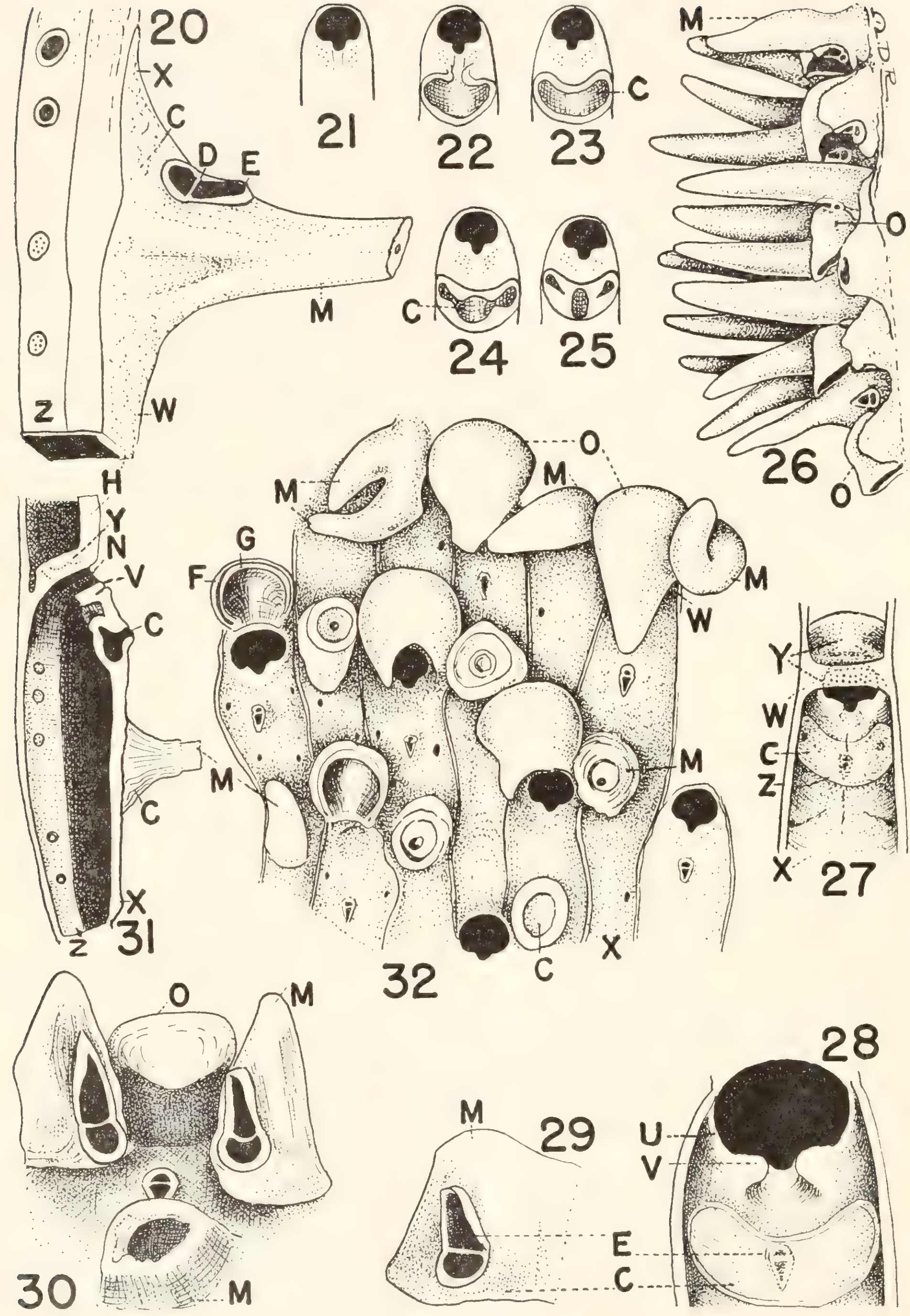
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PLATE III.

ened and complete. The ectooecium forms a partial incomplete shallow shell or band about the lateral and distal periphery of the entooecium.

Areolae occur in the ectooecium. Faint depressions emanate from these areolae. The ectooecium grows upward from around and between them to form the band about the entooecium. At the advancing border of the ectooecium the entooecial surface appears tucked or crowded because of the peculiar growth method or encroachment of the ectooecium. Not enough material was available for a more detailed study of the ovicell.

Distribution and ecology. A flat rock, measuring roughly about 17 cm. long, 10 cm. wide and 3 cm. thick, well encrusted with a dozen species of bryozoa and sponge, contained four small patches of *Toretocheilum absidatum.* These were on the side of the rock and colony fragments had to be chipped off or calcined off for study under the compound microscope.

The rock came from a depth of 40 fathoms, from USN Sta. 240 of Marguerite Bay, Antarctica, Comdr. D. C. Nutt collector. The specimens will be deposited

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PLATE III

All figures on this plate are of *Isoschizoporella tricuspis.* All except Figures 21 through 25 are drawn with a camera lucida. Figures 20, 26, 29 and 32 are from Sta. 226 material. The rest are from Sta. 234.

FIGURE 20. Portion of zooecium, showing aviculiferous spine, frontal and lateral walls. The lateral wall has two pore plates (the two lower porous discs) and the "corresponding openings" (the two upper doughnut-shaped discs). The pore plates of one zooecium line up with the corresponding openings of its neighbor zooecia and vice versa.

FIGURES 21 THROUGH 25. Diagrams of external surface of frontal wall, depicting stages in the formation of the suboral avicularial chamber.

FIGURE 21. Zooecial front before an avicularial chamber has begun to form.

FIGURE 22. The avicularial chamber is outlined by a growing calcareous rim. The border is still incomplete distally.

FIGURE 23. Avicularial chamber now completely outlined.

FIGURE 24. Borders of the avicularial chamber growing and approximating.

FIGURE 25. Roofing over and fusion stage of avicularial chamber formation. Three open spaces still remain but these will be reduced in time to two areolar pores and an avicularium.

FIGURE 26. Side view of a spiny patch of colony with hooded ovicells between the spines. The spines at extreme right are either low or broken off and the ovicells can be seen just beyond them.

FIGURE 27. Back wall removed to show the interior of the front wall and the relative position of the distal wall. The distal wall with its sieve plate hides half the orifice. Cardelles and avicularial chamber also are visible from this side.

FIGURE 28. Enlargement of inner surface of orifice, cardelles, peristomial and vestibular sinuses and avicularial chamber. The avicularium shows through the translucent wall.

FIGURE 29. Early stage in the development of an aviculiferous spine. At this stage the avicularium and its chamber are present and the spine will result from the growth of the tip of the mound.

FIGURE 30. A more advanced stage in the growth of an aviculiferous spine. Two such spines flank a broken ovicell. Between their base lies a suboral avicularium. In the foreground is an exposed avicularial chamber of another aviculiferous spine (broken off).

FIGURE 31. Sagittal section through a zooecium. The darkest areas represent the zooecial and avicularial cavities. The upper three circles in the gray lateral wall are the pore plates. The two lower circles are the "corresponding openings" which would fit next to the pore plates of a neighbor zoid.

FIGURE 32. An ovicelled, spined, well calcified section of a colony. The upper tier of zooecia shows a curved spine and a double spine. The four spines of the middle and lower tiers are broken off. Ovicell shapes are variable. Two left ovicells are broken off and reveal the double calcareous wall.

with the Smithsonian Institution, U. S. National Museum, after the other species on the rock have been thoroughly studied.

GENUS *ISOSCHIZOPORELLA,* NEW GENUS

Diagnosis. Orifice suborbicular, with V-shaped sinus in its proximal lip. The two ends of the C-shaped vestibular arch stop at the sinus and form ledges (cardelles) to support the operculum. Operculum has two muscle dots distally and a proximal median tab to fit the sinus. Frontal wall smooth to beaded, flattened except for avicularial chamber and bordered by faint mural rim. Median suboral avicularium with mandible usually proximally directed present some distance below orifice. The reniform, bilaterally symmetrical avicularial chamber extends across the entire front wall and has a lateral pore on each side usually. Other avicularia are present in association with stout umbonate spines or tumid mounds. Frontal wall a pleurocyst, punctured by only a few peripheral pores spaced far apart and with bilateral symmetry. Ovicell globose to hood-like, smooth to granulated, imperforate, with double calcareous wall. Ovicells may grow downward over orifice.

Name derivation. The name *Isoschizoporella* was coined from the Greek word *isos* meaning like or equal and the already long existing generic name *Schizoporella* which was derived from *schizo* (to divide), *poros* (pore) and *ella* (diminutive).

Genotype. The genotype, here chosen for the new genus *Isoschizoporella,* is *Schizoporella tumida* var. *tricuspis* Calvet 1909, here elevated to species rank: *Isoschizoporella tricuspis* (Calvet).

Remarks. The reason for the erection of this new genus was two-fold. Firstly, there was need to set up a taxon for a troublesome species which had a schizoporellid orifice but which possessed other features which disqualified it from the presently restricted family Schizoporellidae. Secondly, the troublesome species seemed to belong to the family Hippoporinidae but possessed a totality of characters that would not permit its uncontested inclusion in any presently known hippoporinid genus. So it was thought best to erect a new genus for it, since this is a very rare form restricted to the Antarctic and sub-Antarctic regions.

The character by which *Isoschizoporella* differs from the family Schizoporellidae and resembles family Hippoporinidae is the nature of its frontal wall. *Isoschizoporella* has a pleurocyst, as does the family Hippoporinidae, while the Schizoporellidae have a tremocyst. A pleurocyst is a frontal wall that is granular, imperforate over the central area and perforated only around the periphery by areolar pores. A tremocyst is a frontal wall usually liberally sprinkled all over with pores.

The *Isoschizoporella* pleurocyst has a few elongate inconspicuous peripheral pores, very widely spaced, usually placed with bilateral symmetry at zooecial corners, and some elsewhere en route, like the paired pores of some Reteporidae as *Iodictyum* (Harmer, 1934; pp. 515, 522) in particular. The *Hippadenella carsonae* (Rogick, 1957b; Plate I) frontal is almost identical with that of *Isoschizoporella* but the orifice, operculum and ovicells are different.

Synonymy and previous records:

1909. *Schizoporella tumida* Hincks 1881, var. *tricuspis.* Calvet, pp. 28–30; Pl. III, Figs. 1–3. Excellent description and figures showing ovicelled and non-ovicelled zooecia, operculum and suboral avicularial mandible. No measurements given. From 30 meters' depth at Booth Wandel Isle and 110 meters' depth in Biscoe Bay.
1924. *Schizoporella tumida* var. *tricuspis.* Thornely, p. 12. Commonwealth Bay, Sta. 1, Lat. 66°50′ S., Long. 142°6′ E. at 354 fathoms.
1928. *Schizoporella tumida* var. *tricuspis.* Livingstone, pp. 7, 52.
1957a. *Schizoporella tumida* var. *tricuspis.* Rogick, p. 8, USN Sta. 226, Marguerite Bay. Ecological note.

The above are the only records of this species up to date.

Diagnosis. Colony bilaminate, foliaceous; smooth except for spiny or ovicelled patches. Zooecia long, narrow, flattened, bracket-shaped. Mural rims thin, faintly salient. Frontal a granulated or beaded pleurocyst with a few paired peripheral pores. A small median oval to pointed suboral avicularium sits atop the wide immersed reniform avicularial chamber. A stout tusklike aviculiferous process ("spine") occurs on the midfrontal of zooecia adjacent to an ovicelled zoid. Some zoids without spines or ovicells. Ovicell imperforate, granular, shaped like an elephant's head, overhanging the zooecial orifice, usually flanked on each side by its neighbors' aviculiferous frontal spines. Zooecial orifice and operculum as in genus. The median V-shaped peristomial sinus is less than half the width of the proximal lip. Zooecial orifice is at distal end of frontal and is touched by the frontals of the three adjacent zoids. Distal to orifice, at the beginning of the next zoid is a membranous area, crescent-shaped. Zooecial end wall a sieve plate. Four or five multiporous pore plates or corresponding openings present in each lateral wall.

Measurements. All readings are in millimeters. H is for height; D is for diameter at base.

1.181–1.991	(1.506)	L	zooecia, 20 readings
0.272–0.432	(0.351)	W	zooecia, 20 readings
0.144–0.173	(0.158)	L	orifice, including sinus
0.173–0.202	(0.184)	W	orifice
0.143–0.176	(0.163)	L	operculum
0.166–0.195	(0.182)	W	operculum
0.446–0.619	(0.531)	L	ovicell, including its beak
0.317–0.461	(0.413)	W	ovicell
0.072–0.115	(0.093)	L	suboral avicularium
0.043–0.072	(0.055)	W	suboral avicularium
0.156–0.234	(0.190)	L	spinal avicularium, 8 readings
0.091–0.137	(0.107)	W	spinal avicularium, 8 readings
0.760–1.177	(1.039)	H	frontal spine
0.377–0.514	(0.456)	D	at widest part of spine base avicularial chamber
0.260–0.358	(0.311)	D	at narrowest part of spine base avicularial chamber
0.046–0.078	(0.061)	L	mandible of suboral avicularium

0.039–0.065	(0.051)	W mandible of suboral avicularium
0.117–0.130	(0.124)	L mandible of spinal avicularium, 2 readings
	(0.130)	W mandible of spinal avicularium, 1 reading

Remarks. Calvet's hesitancy (1909, p. 28) about the identity of his *tricuspis* and its exact relationship to *Schizoporella tumida* Hincks (1881, p. 13, Pl. I, Fig. 3) is understandable in view of the brevity and incompleteness of Hincks' description and illustration. Hincks' figure shows four non-ovicelled zooecia and a mound bearing a special avicularium. This mound is described by Hincks as "frequently an ovate rising on the side of the cell extending from the orifice down a considerable portion of its length, bearing on its upper extremity an immersed avicularium, with pointed mandible directed downwards." The *S. tumida* ovicell is described as "globose and prominent, with a smooth surface."

Hincks neither mentions nor figures the frontal pores or frontal spines and the ovicell is apparently without the downward orifice-covering beak. Also, the position of the suboral avicularium is much closer to the orifice in *S. tumida* than in *Isoschizoporella tricuspis*. *Schizoporella tumida* Hincks was collected in the Bass Straits of Australia, while *Isoschizoporella tricuspis* is known only from the Antarctic. In these ways then does *S. tumida* differ from Calvet's var. *tricuspis*, so it is best to elevate Calvet's variety to species rank.

Colony. Colony color is light écru. Judging from the fragments the colony should be good sized, foliaceous or fan-shaped, with crinkled edges. In the USN collection were a number of chips or fragments, none of them a complete colony. The largest piece was 67 mm. long by 60 mm. wide, and probably represented less than half a colony because it was a portion of the growing edge, apparently some distance away from the base of the colony, judging by its width.

Some of the pieces have occasional faint markings, like growth rings (Fig. 19). New linear or radial rows of zooecia are added in such zones, or when needed, to make possible the ever widening periphery or ruffled edge of the colony.

Colonies are bilaminate and solid, with zooecia back to back, and mostly smooth. Occasional bristly patches occur on either face (Figs. 13, 16, 19, 26). These patches represent areas where ovicells and aviculiferous spines have arisen. One such patch was 12 mm. long by 10 mm. wide.

Sometimes secondary calcification closes over the zooecial orifices (Fig. 16) but the suboral avicularia and frontal pores seem much more resistant to secondary filling-in. The secondary calcification seems to begin with the operculum itself. It fills in or frosts over lightly, then more solidly till the whole orifice is like the surrounding frontals.

Colonies are relatively clean, living zoids apparently being resistant to encrustation with extraneous material, but dead parts show some settlers.

Soft parts. Many zoids had opercula and mandibles. Far fewer contained polypides (gut, tentacles, musculature). These polypide remains, when present, were very slim, in keeping with the slenderness and elongation of the zooecia and also perhaps the food supply was insufficient. Some of the zoids had the opercula fouled or rimmed about by a border of scummy orange-colored material, as if wastes had accumulated about the orifice and killed the zooecia. It was not possible to determine if embryos were present in ovicells because the ovicells are so opaque.

Suboral avicularium. A suboral avicularium develops on nearly every zoid. It is median, oval, with a triangular mandible that is proximally directed (*cf.* Figs. 11, 14). Most of the suboral avicularia are so oriented longitudinally but occasional ones do occur a bit off course. That is, their slant may be slightly oblique, to right or left. One colony fragment from USN Sta. 234 contained a zoid whose suboral avicularium pointed transversely while the rest of the suboral avicularia in that fragment were oriented in the usual manner.

Sometimes the mandibular area tip (rostrum) is flat against the frontal ("chest"), other times its tip is more elevated.

The suboral avicularium forms after its reniform chamber is complete (Figs. 11, 21–25, 27, 28, 31). The floor of the chamber is the zooecial frontal wall. The chamber stretches from lateral wall to lateral wall. The chamber walls grow upward from the zooecial frontal, converge in a transverse line that has three diminishing gaps in it (Figs. 22–25). These gaps are the two lateral pores and the median avicularium. The mandibular area, back area and pivot develop over the median gap. At the base of the chamber, particularly on the lateral side, can be found the aforementioned pore and perhaps another one, puncturing the chamber.

Aviculiferous spines. The big tusk-like frontal spines are present on some zoids, either ovicelled or non-ovicelled, and absent from others. They are aviculiferous, *i.e.*, house an avicularium and avicularial chamber in their base (Figs. 13, 20, 30, 31). When present, there is generally only one spine per zoid. An occasional spine may be forked (Fig. 32) but most of them are not. Some of the spines are straight, some curved. They are tall, thick-walled, wide at the base and taper to a blunt tip and sometimes look corrugated. They are hollow, the basal part being especially large (Figs. 20, 31). The cavity of the spine is not continuous with the cavity of the zooecium. The basal part of the spine cavity is the avicularial chamber.

These spines arise as calcareous blisters on the midfrontal wall of a zoid (Figs. 31, 32). They sprout close to a neighbor's orifice and ovicell as if to protect both (Figs. 16, 32). One wonders what influence, if any, the developing ovicell or developing embryo of a zoid exerts on neighboring zoids to cause them to produce the protective aviculiferous spines.

When still in the first stage of formation the avicularial spine base is very extensive. One was 0.247 mm. wide by 0.52 mm. long, as wide as some of the zooecial fronts. The base is oval or reniform in outline. Strictly speaking the spine base represents the avicularial chamber.

The avicularium at first is oblique in position like a door leaning against a mound. But, with the continued upward growth of the mound to form a hollow spine the position of the avicularium shifts until it is roughly vertical, *i.e.*, at right angles to the zooecial plane. The avicularium appears as if propped up against or incorporated into the proximo-lateral side of the spine. Its duckbill-shaped mandible (Fig. 15) is larger than the triangular mandible of the suboral avicularium. Also, the suboral avicularium itself is smaller than the spine avicularium. The mandible of the spine avicularium is directed toward the tip of the spine (Figs. 20, 26).

In the USN material these aviculiferous spines develop in the vicinity of ovicells or orifices of ovicelligerous zoids, leaning toward the ovicells. Generally an ovicell has an aviculiferous spine on each side of it (Figs. 13, 16) but there can be exceptions. Occasional ovicells may share a spine between them. Still others may have but a single spine beside them.

Interzoidal communications. In *I. tricuspis* the end walls of zooecia are generally single partitions or transverse septa between succeeding zoids in a linear or radial series, *i.e.*, two zoids share the same single end wall between them. The lateral walls are double, each neighboring zoid having its own individual lateral wall. Therefore it is not surprising that the interzoidal communications between zoids should be different in the two different kinds of walls. Silén (1944) made an extensive study of this situation in a number of different species.

In *I. tricuspis* the transverse and the lateral walls are perforated, but differently. The end wall is truncated, *i.e.*, divided into two sections, one with the porous sieve plate, the other without pores (Figs. 17, 18, 31). The sieve plate is nearer the basal wall than is the non-porous section and slants away from the basal wall at about a 45° angle. The other section is more nearly vertical with respect to the basal wall. The number of closely set pores in the slanting sieve plate is hard to count but one zoid had about 16, more or less.

The lateral wall has 4 or 5 multiporous pore chambers or corresponding openings (Figs. 20, 31). These blister-like pore chambers have fewer pores, about 3 to 8, than do the sieve plates.

Distribution and ecology. A small number of fragments, about 18, and none of them a complete colony, of *Isoschizoporella tricuspis,* were collected by the 1947–1948 USN Expedition from two stations in Marguerite Bay, Antarctica, Sta. 226 and 234, from depths of 40 fathoms. These localities are near that from which Calvet's specimens came. The Thornely specimens were collected at opposite sides of the Antarctic continent and from a considerably greater depth, 354 fathoms. So, although the species showed up in very small quantity in the collections of only three Antarctic expeditions, its occurrence at opposite sides of the pole would suggest a circumpolar distribution.

The USN specimens were relatively clean of encrustation by other forms but a few bryozoans did gain a foothold on some of the fragments. One *Beania erecta* zoid and a lichenoporoid cyclostomatous bryozoan colony, to be identified later, grew on the Sta. 226 fragment. Species found on the Sta. 234 fragments included the bryozoans *Osthimosia milleporoides* (Calvet) 1909, *Phylactellipora lyrulata* (Calvet) 1909 and *Ramphonotus inermis* (Kluge) 1914 and foraminifera.

The *I. tricuspis* specimens will be deposited with the Smithsonian Institution, U. S. National Museum.

SUMMARY

1. Two new genera, *Toretocheilum* and *Isoschizoporella,* of the family Hippoporinidae (Bryozoa, Cheilostomata) have been erected on the basis of their orifices and areolated frontal walls.

2. One new species, *Toretocheilum absidatum,* was described. Another, *Schizoporella tumida* var. *tricuspis* Calvet 1909, was elevated to species rank and transferred to a new genus: *Isoschizoporella tricuspis.*

3. Both species are amply illustrated, measurements given and the description for *I. tricuspis* has been amplified. Attention is given to the range of variation of each species.

4. Each species is designated as the genotype of its new genus. Both species are from Marguerite Bay, Antarctica.

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DISCONTINUOUS RESPIRATION IN INSECTS: ROLE OF THE SPIRACLES[1]

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In many groups of insects[2] metabolic carbon dioxide is retained within the insect and released during brief periods in "bursts" (Punt, 1944, 1948, 1950a, 1950b, 1956; Schneiderman, 1953; Schneiderman and Williams, 1953–1955; Buck *et al.*, 1953; Buck and Keister, 1955; Ito, 1954). In diapausing pupae of the Cecropia silkworm, for example, more than nine-tenths of the metabolic carbon dioxide is stored and then released in brief bursts, which occur from once every week to many times per hour, depending on the temperature and metabolic rate. The remaining carbon dioxide escapes during the interburst period. When measured by usual manometric procedures, the uptake of oxygen, unlike the release of carbon dioxide, appears continuous and almost steady (Schneiderman and Williams, 1953a, 1955; Buck and Keister, 1954, 1955). If the spiracles are sealed with paraffin, virtually all the respiratory exchange ceases (Ito, 1953; Schneiderman and Williams, 1955); the spiracles are, therefore, the site of both the discontinuous release of carbon dioxide and the simultaneous continuous uptake of oxygen. When metabolic rate is low, the bursts are accentuated. Thus at 10° C. carbon dioxide may be given off only once a week and the interburst rate of carbon dioxide may be but 1/100th the rate of oxygen uptake. This indicates the true dimensions of the respiratory paradox: oxygen enters the spiracles during the interburst period at many times the rate at which carbon dioxide leaves and, furthermore, the insect releases its carbon dioxide periodically.

The central importance of spiracular behavior in the discontinuous release of carbon dioxide was suggested by the observation of Buck and his co-workers (1953, 1955) that intubating the spiracles of *Agapema* pupae eliminated the bursts of carbon dioxide. Subsequently, we observed that excision of the valve from one of the fourteen functional spiracles of a Cecropia pupa caused carbon dioxide output to become continuous (Schneiderman and Beckel, 1954; Schneiderman, 1956). The only maneuver that restored the discontinuous release of carbon dioxide was the sealing of the open spiracle. It appears from this experiment that all the spiracles

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[1] This research was aided by a research grant, H-1887, from the National Heart Institute of the United States Public Health Service.

[2] Groups include larval and adult Hemiptera (*Rhodnius prolixus; Triatoma rubrofasciata, Cimex lectularius;* adult Dictyoptera (*Periplaneta americana*); larval and adult Orthoptera (*Locusta migratoria*); adult Coleoptera (*Carabus nemoralis, Meloë proscarabaeus, Hadrocarabus problematicus*); diapausing larvae of Lepidoptera (*Arctia* sp.); diapausing pupae of Lepidoptera (*Hyalophora cecropia, Antheraea polyphemus, Samia cynthia, Rothschildia oryzaba, Agapema galbina, Bombyx mori* ("dauer pupae"), *Sphinx ligustri, Agrotis* sp., *Papilio machaon*); non-diapausing pupae of *Bombyx mori*, diapausing adults of Lepidoptera (*Vanessa urticae*).

remain nearly closed during the interburst, preventing carbon dioxide from diffusing out in substantial quantities, and that one or more of the spiracles opens during a burst. The spiracular valves thus hold an important key to the discontinuous release of carbon dioxide.

Several theories have been proposed to explain the disparate rates of oxygen and carbon dioxide transfer during the interburst period, as well as the bursts themselves (Punt, 1944, 1950a; Punt *et al.*, 1957; Zeuthen, 1955; Buck *et al.*, 1953; Buck and Keister, 1954, 1955; Schneiderman, 1956). The most recent and comprehensive theoretical analysis of the entire phenomenon is that of Buck (1958a, 1958b). This theory and all of the others depended for the most part upon (a) indirect estimations of tracheal carbon dioxide and oxygen tensions during the "burst cycle," (b) postulated behavior of the valves that regulate the opening of the spiracles, (c) assumed changes in intratracheal barometric pressure for which there was no empirical evidence whatever, (d) hypothetical changes in the volume of the tracheal system, (e) cataclysmic biochemical changes. To test these theories and to resolve the paradox, it proved necessary: (a) to continuously record the behavior of the spiracular valves during the burst cycle, (b) to measure directly the changing composition of tracheal gases during the cycle, (c) to measure the changing intratracheal barometric pressure, and (d) the changing tracheal volume during the cycle. The present report initiates a series in which methods will be described that accomplish these objectives and provide fairly precise pictures of both the partial and absolute pressure gradients driving oxygen into the insect, the gradients driving carbon dioxide out of the insect, and cyclic variations in the aperture of the spiracles and in the volume of the tracheal system. The results to be reported confirm that the breathing of silkworm pupae involves processes other than physical diffusion, and also bear out many of the theoretical predictions of Buck (1958b). However, in some essential points, they do not support his theory, but instead provide evidence for another kind of insect breathing which seems different from any previously proposed or demonstrated and which may account for discontinuous respiration. In addition, they define the stimuli that cause the cyclical activity of the spiracles.

The first paper focusses on the role of the spiracles in discontinuous respiration. A preliminary account of some of these results has been given elsewhere (Schneiderman and Beckel, 1954; Schneiderman, 1956).

MATERIALS AND METHODS

1. *Experimental animals*

Experiments were performed on diapausing pupae and developing adults of the giant Saturniid silkworms *Hyalophora cecropia*, *Antheraea polyphemus* and *Samia cynthia*. In our experience these species of closely related moths behave in virtually identical fashion in the sorts of experiments that were undertaken. Animals were reared on net-covered trees or purchased from dealers and stored at 25° C. and 60% to 70% relative humidity. All experiments were conducted at 25° ± 1° C.

2. *Respiratory measurements*

Respiratory exchange was determined manometrically by techniques previously described (Schneiderman and Williams, 1953a, 1955). Measurements were per-

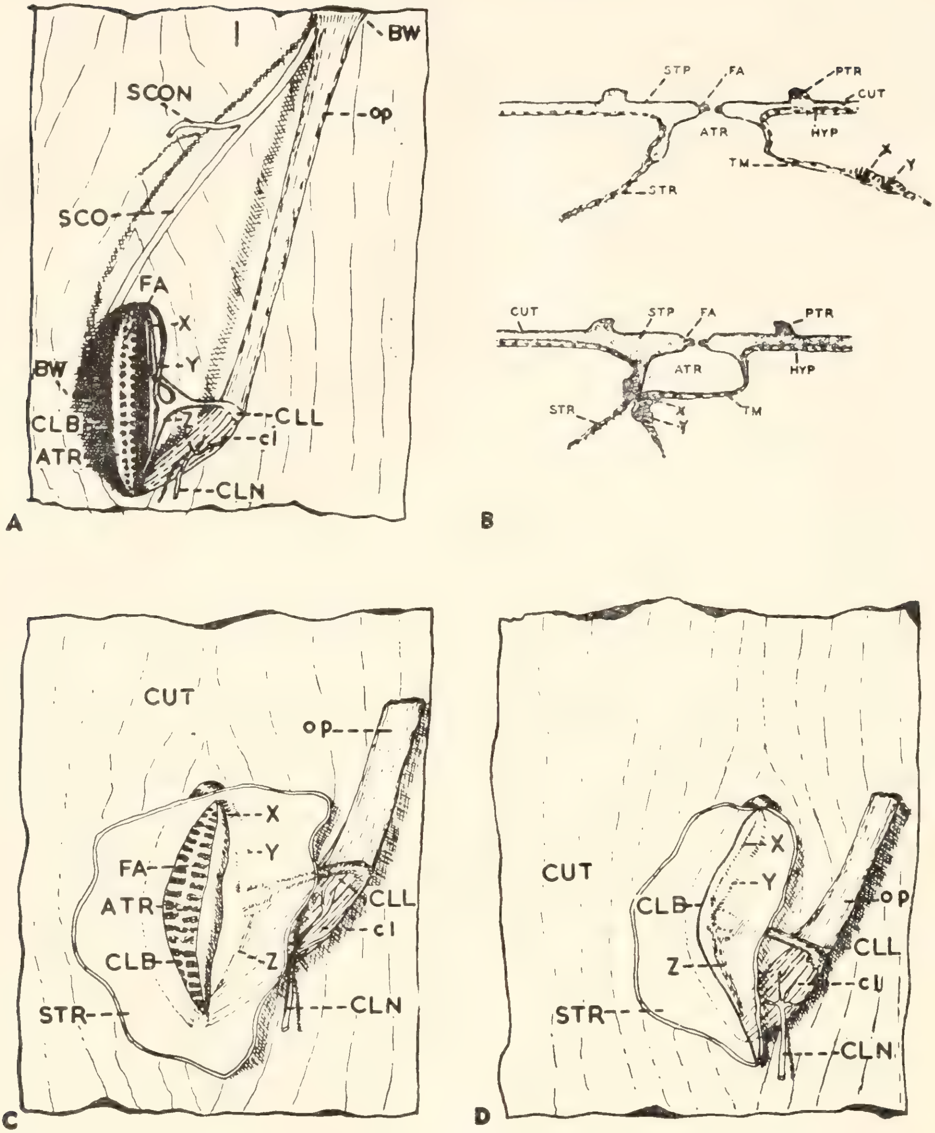
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FIGURE 1. (A) Pupal spiracular regulatory apparatus of Cecropia, as seen from inside the animal. No tracheae are shown. *ATR*, atrium; *BW*, body wall; *cl*, closer muscle; *CLB*, closing bow; *CLL*, closing lever; *CLN*, nerve of closer muscle; *FA*, filter apparatus or stigmal plate; *OP*, elastic opener; *SCO*, scolopophorous organ; *SCON*, nerve of scolopophorous organ; *X*, dorsolateral closing bar; *Y*, dorsomedian closing bar; *Z*, ventral closing bar. (B) Frontal sections through the spiracular region, above the closing lever, and cutting the two dorsal closing bars. These show how the spiracle closes when the closing bars are pushed against the closing bow or atrium. *CUT*, cuticle; *HYP*, hypodermis; *PTR*, peritreme; *STP*, stigmal plate, *STR*, spiracular tracheal manifold; *TM*, tracheal membrane. (C) Apparatus viewed from the inside with the scolopophorous organ and part of the elastic opener removed. Only part of the spiracular tracheal manifold is shown. The valve is open. (D) Same as (C) only the valve is closed.

formed by the "direct manometric method" (Umbreit *et al.*, 1958) in 45-cc. cylindrical vessels equipped with venting plugs and adapters, for use with standard Warburg manometers.

3. *Recording of spiracular movement*

(a) *Anatomy of the spiracle*

These silkworm pupae have a pair of thoracic spiracles and six pairs of functional abdominal spiracles. Each spiracle is surrounded by a chitinous peritreme, and covered by a stigmal plate, or filter apparatus, which communicates by a thin slit to a chamber below—the atrium—which contains the spiracular valve. Gas exchange between the atrial chamber (and hence the atmosphere) and the interconnecting tracheal system, which lies just below it, is regulated by this valve. The morphology of the spiracular apparatus and some of the physiological properties of the spiracular muscle of pupae of Saturniid moths have been described most recently by Beckel (Beckel, 1955, 1958; Beckel and Schneiderman, 1956, 1957). For our present purposes, it is sufficient to note that the spiracular valve is an epithelial membrane which is firmly attached to a chitinous frame consisting of a bow and three bars which unite in the middle to give rise to a lever (Fig. 1). A closer-muscle stretches from the ventral tip of the lever to the ventral corner of the valve. It is opposed by an elastic ligament which extends from the dorsal tip of the lever to the body wall. When the closer muscle contracts, it pulls on the lever and closes the valve. When it relaxes, the valve opens because of the elasticity of the chitinous frame and the tension of the opposing elastic ligament. The muscle is innervated by a nerve from the corresponding segmental ganglion and by a branch of the median nerve of the next anterior segment.

(b) *Recording valve movements*

To expose the valves in the living insect it was necessary to remove the overlying chitinous stigmal plate. This could easily be done under the dissecting microscope after the animal had been anaesthetized with carbon dioxide. Insofar as could be judged, this operation in no way interfered with the normal functioning of the valves, which were now clearly visible. When the valves were so exposed for a week or more, they occasionally dried out and ceased functioning normally. To prevent this, after each period of observation, the spiracular opening was sealed with Tackiwax.[3] Because of their greater accessibility, the abdominal spiracles were examined in preference to the thoracic pair. Of these the first, second, and third abdominal were the easiest to study because they served non-collapsible segments and were not obscured when the animal moved its abdomen.

Several methods for recording spiracular movements of intact insects have been employed in the past and there have been several descriptions of spiracular behavior in the cockroach *Periplaneta americana* (Hazelhoff, 1926a, 1926b), the phasmid *Dixippus morosus* (Stahn, 1929); the rat flea *Xenopsylla cheopis* (Wigglesworth, 1935; Herford, 1938), the bedbug *Cimex lectularius* (Wigglesworth, 1941), the grasshoppers *Dissosteira carolina* (McCutcheon, 1940), *Schistocerca obscura* (Watts, 1951), and *Schistocerca gregaria* (Hoyle, 1959), the larva of the commercial silkworm (Shimizu and Ono, 1942), the flies *Musca domestica* and *Callitroga macelleria* (Case, 1956a) and the cockroaches *P. americana* and *Blaberus craniifer* (Case, 1957b). The older literature has been summarized by Hazelhoff (1926a). The present experiments employed a modification of the ocular micrometer system of McCutcheon. A pupa with one or more of its spiracles exposed to view was placed beneath a binocular dissecting microscope furnished with an ocular micrometer and examined under 60 ×. The hairline of the micrometer was focussed on the leading edge of the spiracular valve and was adjusted to follow the valve as it opened and closed (Fig. 2). The crosshairs on the micrometer eyepiece indicated the position of the hairline when the valve was closed. The hairline was moved by a rotating knob whose action was translated into a kymograph trace by means of a system of pulleys and levers and an ink-writing pen recording on a

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[3] After the present experiments were completed, we discovered that a valve can be prevented from drying out by sealing a small transparent plastic window over the exposed spiracle. Although the window prevents gas exchange through the spiracle, it allows convenient observation of spiracular movements, and the valve functions for many weeks.

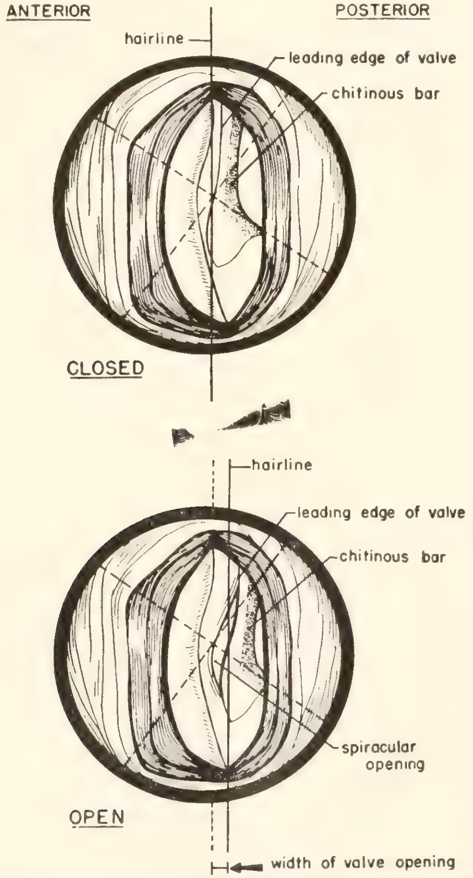


FIGURE 2. Spiracle as seen through ocular micrometer. The filter apparatus and most of the stigmal plate have been removed and the valve is visible. For further details see text.

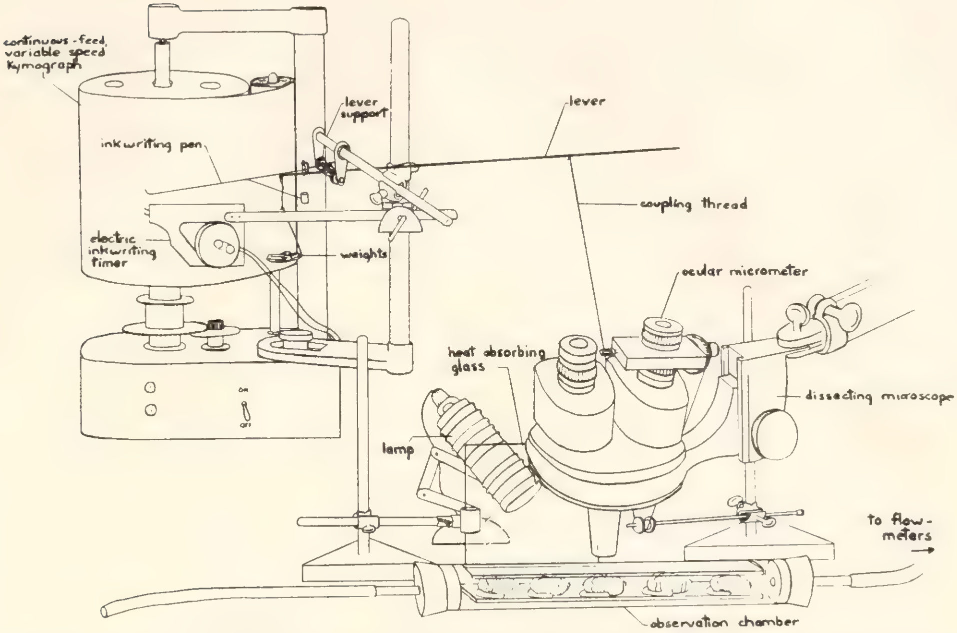

continuous-feed, variable speed kymograph
lever support
lever
inkwriting pen
coupling thread
electric inkwriting timer
weights
ocular micrometer
heat absorbing glass
dissecting microscope
lamp
to flow-meters
observation chamber

FIGURE 3. Apparatus used for recording spiracular valve movements. The observation chamber shown is of the sort also employed in studies with intubated pupae. For further details see text.

continuous feed kymograph (Fig. 3). The tracings of the ink-writing pen provided a record of the movements of the spiracular valve, each deflection on the record representing a valve movement. The ordinate of the trace shows the width of valve opening in arbitrary units, while the abscissa denotes the duration of the opening. Directly beneath the record of valve movements the time was recorded by an ink-writing timer.

In most of the experiments spiracular behavior was viewed through a 20-cc. glass chamber in which the insect was held secure in a plasticene support, or in a 250-cc. lucite chamber with a flat surface for optical convenience. Air and gas mixtures were flushed through the chamber as desired. The rate of gas flow varied from 25 to about 500 cc./min. in different experiments and appeared to have no effect on the phenomena under investigation. The time required to change the atmosphere in the small chamber was rarely longer than 10 seconds, but for the large chamber it took about two minutes.

Gas mixtures were either pre-mixed in pressure cylinders or made up by proportional flow. All mixtures were analyzed periodically.

A. EXPERIMENTS WITH INTACT PUPAE

1. *Normal behavior of spiracles*

Figure 4 is a portion of a typical record of the activity of the third abdominal spiracle of a Cecropia pupa over a period of four hours. A consistent pattern of valve movement occurred in cycles lasting 45 minutes to 2 hours. After a period of about 10 minutes, during which it remained closed and motionless, the valve fluttered for between 15 and 40 minutes and then, within a minute, the valve movements progressively increased in amplitude until the valve opened fully. Swaying slightly, it remained open for several minutes. Then it alternately opened and closed for several minutes, and the valve movements gradually decreased in amplitude and duration, until the valve closed altogether. Following this, the valve remained motionless until the fluttering preceding the next period of wide openings. This cycle was repeated over and over, and apparently represented the normal behavior of the spiracle.

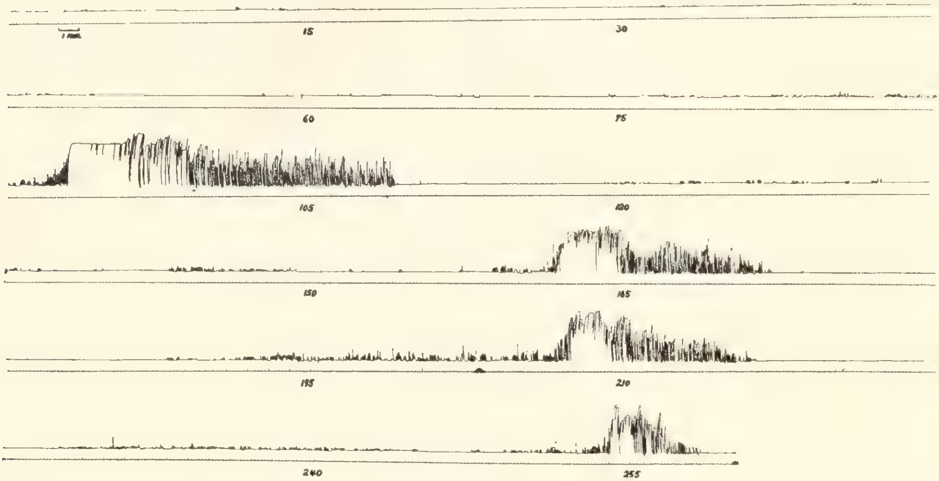


FIGURE 4. Record of the valve movements of a third abdominal spiracle of a diapausing Cecropia pupa over a 4-hour period in air at 25° C. Each mark above the baseline represents a valve opening. Occasional marks below the baseline are artifacts caused by vibration of the apparatus.

Manometric observations of the carbon dioxide output of this pupa revealed bursts of carbon dioxide at intervals of 30 minutes to two hours. A similar correlation between the frequency of carbon dioxide bursts and periods when the valve was widely open has been consistently observed in all other silkworm pupae studied. Moreover, by utilizing techniques which permit simultaneous recording of both valve movements and gas exchange, it has been possible to show the correlation directly (Schechter and Schneiderman, unpublished observations). Hence it seems evident that the periodic bursts of carbon dioxide result from the periodical prolonged openings of the spiracles, which we have termed "spiracular bursts." The term "burst" seems appropriate for both the spiracular and the manometric events, since they coincide. The spiracular burst can conveniently be partitioned into a period of wide openings—the "open phase"—which is followed by a period of rapid closures—the "decline phase." The end of the decline period and of the spiracular burst is marked by the moment the valve constricts tightly. The "interburst" consists of a "constriction period" after the burst, when the valve appears closed and motionless, and a "flutter period" prior to the next burst.

The flutter period was usually quite irregular in terms of both the frequency and amplitude of the valve movements. As Figure 4 reveals, the flutters usually

TABLE I

Duration of various phases of spiracular burst cycles in a typical series of diapausing Cecropia pupae and developing adults of Polyphemus

Experiment No.***	Cycle length (minutes)	Duration of various phases (minutes)*			
		Interburst		Spiracular burst	
		Constriction	Flutter	Open	Decline
Cecropia					
827	114	13 (11)	91.5 (80)	5.5 (4.8)	4.0 (3.5)
8316	74	10 (14)	61 (81)	1.0 (1.4)	2.0 (2.7)
938	73	26 (36)	42 (57)	1.5 (2.1)	3.5 (4.8)
739	68	26 (38)	37.5 (55)	1.0 (1.5)	3.5 (5.1)
8315	65	12 (19)	49 (75)	1.0 (1.6)	3.0 (4.7)
625 a	63	10 (16)	43 (68)	3.0 (4.8)	7.0 (11.1)
8314	50	16 (32)	29.5 (59)	1.5 (3.0)	3.0 (6.0)
625 b	44	16 (36)	19 (43)	2.5 (5.7)	6.5 (14.8)
725 c	43	12 (28)	26 (61)	2.0 (4.7)	3.0 (6.9)
8311 b	39	12.5 (32)	23.5 (60)	1.0 (2.6)	2.0 (5.1)
439	38	17 (45)	16 (42)	1.0 (2.6)	4.0 (10.5)
8311 c	32	18 (56)	12.5 (39)	1.0 (3.1)	0.5 (1.6)
8311 a	29.5	7.5 (25)	19 (65)	1.5 (5.1)	1.5 (5.1)
Average**	56.35 ± 23.25	15.08 ± 5.71	36.12 ± 21.96	1.81 ± 1.28	3.35 ± 1.82
Average %	100	29.85 ± 12.94	60.38 ± 13.67	3.31 ± 1.52	6.30 ± 3.71
Polyphemus					
522 a	16.5	0	15.0 (91)		—
522 b	15.4	0	12.3 (80)	—	—
527 a	10.7	0	6.4 (60)	—	—
527 c	9.7	0	7.0 (72)	—	—
527 d	9.1	0	6.2 (68)	—	—
527 f	8.1	0	5.5 (68)	—	—
5218	7.0	0	4.6 (66)	—	—
113 a	6.0	0	3.5 (58)	1.5 (25)	1.0 (17)
113 b	6.0	0	3.0 (50)	2.0 (33)	1.0 (17)
114	6.0	0	3.6 (60)	1.7 (28)	0.7 (12)
115	5.5	0	2.5 (45)	2.3 (42)	0.7 (13)
521 c	5.5	0	3.5 (64)	—	—
117	4.3	0	2.8 (65)	1.0 (23)	0.5 (12)
521 c	2.7	0	1.4 (52)	—	—
Average	8.04 ± 3.98	0	5.52 ± 3.84	1.70	0.78
Average %	100	0	64.21 ± 11.94	30.2	14.2

* Figures in parentheses record the average per cent of the cycle length.

** ± standard deviation.

*** In this and subsequent experiments, the first two digits refer to the particular animal employed, *i.e.*, all experiments beginning with "82" refer to pupa No. 82.

came in volleys which lasted from 10 seconds to 8 minutes and which were punctuated by closed periods which lasted from 20 seconds to several minutes. Less commonly, flutters occurred singly. Frequency varied from short volleys of about one per second to long volleys of one opening every two to ten seconds. The openings were invariably brief, commonly lasting less than a second. Amplitude also varied somewhat and an occasional wide opening punctuated a series of smaller ones. There appeared to be no systematic variation in amplitude, however, until a minute or two before the burst.

Several hundred hours of records of such cycles have been obtained from about 30 individual pupae. Table I records the duration of the various phases of several typical cycles recorded from diapausing Cecropia pupae and developing adults of Polyphemus. Although the cycles that were studied varied in frequency from 20 per hour to one every two hours, the pattern of valve movements in all cases was remarkably similar. The only variation occurred in pupae with cycles of 15 minutes or less. Here the constriction period was lacking and the spiracular valve was in constant slight motion (see Figures 6 and 7). Also, in pupae with brief cycles the frequency of flutters was considerably greater than in pupae with long cycles, and the spiracular bursts occupied a larger and larger proportion of the cycle.

Simultaneous observations of two or three spiracles on the same side indicated that the spiracles were coordinated. When the valves opened in a burst or closed at the end of a burst, they did so within a minute of each other, though when they fluttered, the pulsations were not in exact synchrony. For our present purposes, these observations indicate that recording the behavior of one spiracle provides an accurate picture of the behavior of all the spiracles of the animal, except possibly the thoracic spiracles whose behavior we have never succeeded in observing.

Previous experiments (Punt, 1950a; Schneiderman and Williams, 1955; Buck and Keister, 1955) pointed out that various factors such as metabolic rate, oxygen and carbon dioxide tensions, etc., profoundly affected the cyclical release of carbon

TABLE II

Burst cycle of a Polyphemus during adult development

Days of adult development	Cycle length (minutes)	Spiracular burst duration (minutes)	Amount of interburst fluttering*
−13	120.0	6.8	+
0	9.8	3.3	++
2	7.0	2.4	++
3	5.0	2.7	++
4	5.4	2.0	++
5	4.4	1.7	++
6	4.7	2.0	+++
9	4.0	1.7	+++
10	4.3	2.0	+++
15	2.7	1.3	++++
16	0	0	++++
20 (emergence)	0	0	++++

* +: Normal amplitude, moderate frequency; ++: normal amplitude, high frequency; +++: greater than normal amplitude, high frequency; ++++: fluttering about half-open position, high frequency.

dioxide. Recognizing the key role of the spiracular valves, it was reasonable to anticipate that these several factors would influence the movement of the spiracular valves. This is clearly shown in the following experiments.

2. *Effects of metabolic rate on the behavior of the spiracular valves*

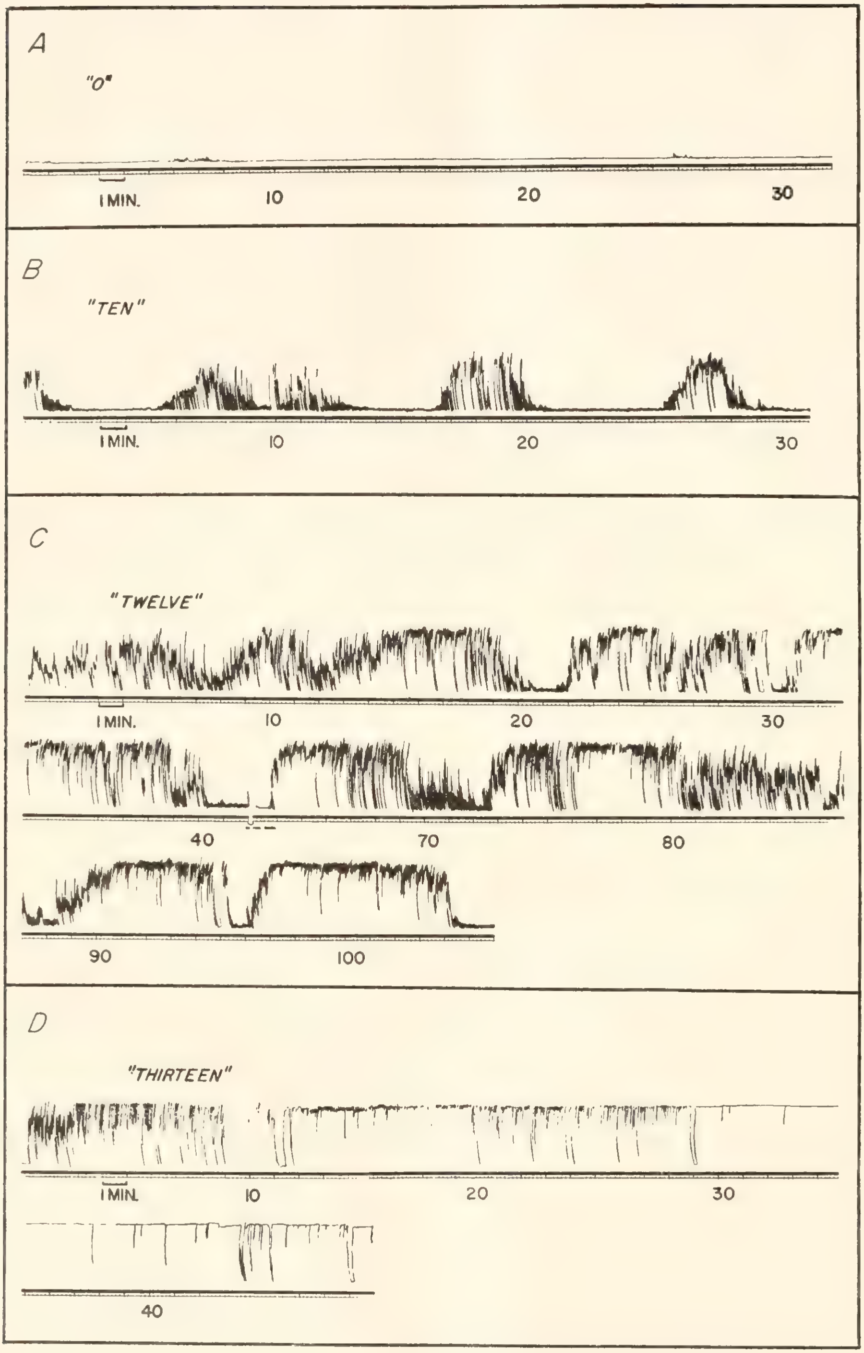
During the pupal-adult transformation, oxygen uptake rises markedly, the cycles of carbon dioxide release become more frequent and the interburst rate of carbon dioxide output increases until the cycles disappear and carbon dioxide is released continuously (Punt, 1950a; Schneiderman and Williams, 1955). Table II summarizes the spiracular behavior of a Polyphemus during adult development, and reveals that the spiracular bursts increased in frequency and that the amount of interburst fluttering also increased, first in frequency and then in amplitude, until eventually on the sixteenth day of the 21-day period of adult development, the spiracular bursts disappeared and the spiracles fluttered continuously about a half-open position. It is important to note that in insects with very high metabolic rates the flutters had far greater amplitude than normal interburst flutters and resembled the spiracular movements encountered in the decline period after a spiracular burst. In any case, by using developing adults, it was possible to obtain animals with brief burst cycles. Another useful means of securing animals with brief cycles for convenient study was to injure pupae. Injury provokes a prompt increase in metabolic rate, which persists for days, and a tremendous increase in burst frequency (Schneiderman and Williams, 1955). In a typical experiment excision of the facial region from a Polyphemus pupa increased cycle frequency from one spiracular burst every two hours before injury, to 12 per hour four days after injury.

The explanation for these effects of metabolic rate on the spiracular burst cycle seems clear. One of the probable consequences of increased metabolic rate is to lower tracheal P_{O_2} and increase tracheal P_{CO_2}. It seems likely that these factors are directly responsible for the acceleration of the burst cycle. This is confirmed in the following experiments on spiracular occlusion.

3. *Effects of spiracular occlusion*

In pupal silkworms, the spiracles are the sole gateways to the tracheal system and their occlusion affords a simple method of lowering internal P_{O_2} and raising internal P_{CO_2}. The effects of spiracular occlusion on the cycle of valve activity of a Polyphemus pupa are seen in Figure 5. Sealing five pairs of abdominal spiracles with paraffin called forth an immediate increase in spiracular burst frequency from about one burst per hour to 6 or 8 per hour. After 12 or 13 of the 14 spiracles were sealed, the spiracular bursts disappeared completely. Unsealing the spiracles initiated the return to substantially normal behavior.

In pupae with five pairs of abdominal spiracles sealed and a spiracular burst frequency of 5 or 10 per hour, it was occasionally possible to restore normal burst frequencies of one to two per hour by exposing the pupa to pure oxygen. The increased oxygen also decreased interburst fluttering and led to wider openings at the time of the spiracular burst. The implications of these observations will be examined in the Discussion. For our present purposes suffice it to say that these spiracular occlusion experiments indicate that low oxygen combined with high carbon dioxide increases spiracular valve movements and speeds up the burst cycle.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20
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40
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80
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100
D
"THIRTEEN"
1 MIN.
10
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40

Figure 5.

TABLE III

Effects of P_{O_2} on the various phases of the spiracular burst cycle of two diapausing Cecropia pupae (S-82 and S-93)

Expt. No.	Ambient P_{O_2}	Average cycle length (minutes)	Mean duration of various phases (minutes) Interburst*				Spiracular burst	
			Constriction	Percentage change in constriction period**	Flutter	Percentage change in flutter period**	Open	Decline
827	21	114	13 (11)	+462	91.5 (80)	−100	5.5 (4.8)	4.0 (3.5)
827a	100	75	73 (97)		0 (0)		2.1 (2.8)	0 (0)
938a	5	Continuous fluttering	—	—	—	—	—	—
939a	15	73	11.5 (16)	−36	58 (79)	+32	1.4 (1.9)	1.5 (2.1)
930 (Av.)	21	66	18.0 (28)	0	44 (65)	0	1.2 (1.9)	3.0 (4.7)
9310	35	50	28 (56)	+56	18 (36)	−59	2.0 (4.0)	2.0 (4.0)
9311	75	61	58 (95)	+220	0 (0)	−100	1.0 (1.6)	2.0 (3.3)

* Figures in parentheses record the *average percentage of the cycle length* occupied by the particular phase. Most of the results represent the average of two cycles except the results in air (930) which represent the average of five cycles of 50 to 74 minutes duration.

** Compared with air controls.

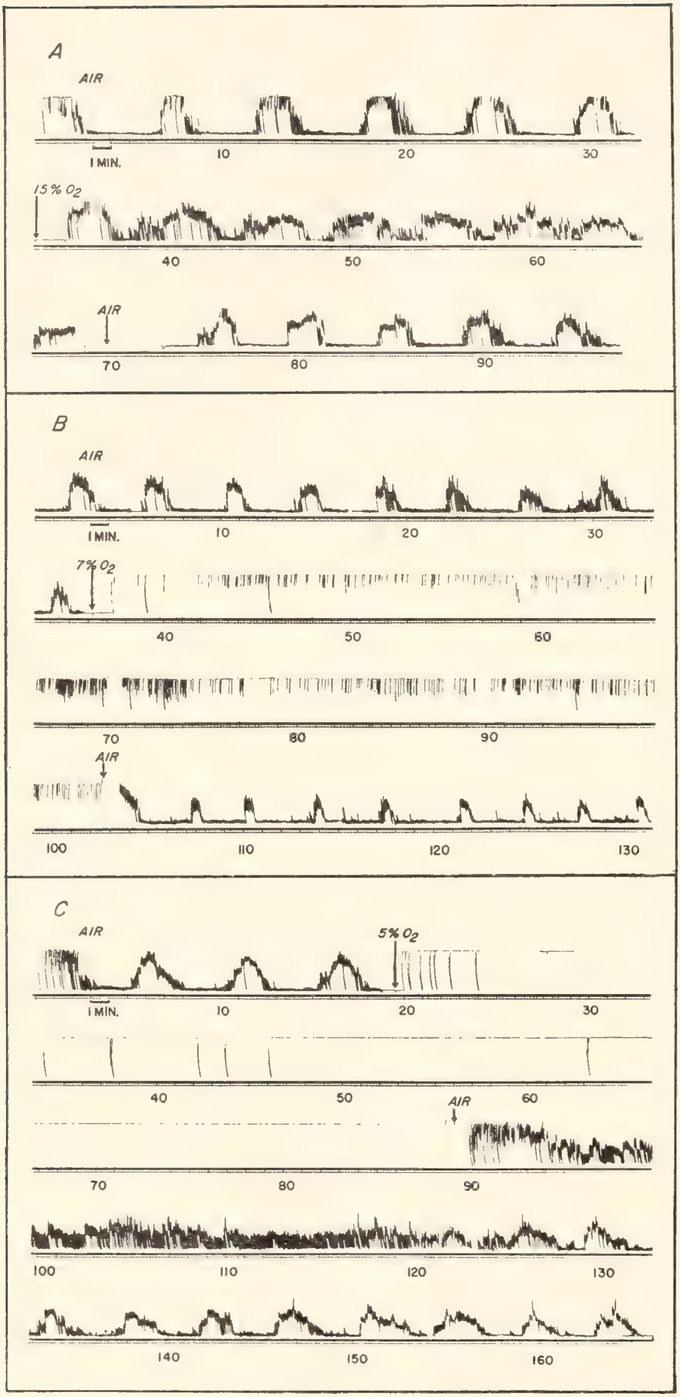
To separate the action of these gases on spiracular behavior, we tested the effects of specific gas mixtures on valve activity.

4. *Effects of oxygen tension on the spiracles*

The effects of decreased oxygen tension on spiracular behavior are evident in Figure 6. This pupa had a high metabolic rate and a brief burst cycle. As Figure 6A shows, within six minutes of lowering the ambient oxygen tension to *15%*, interburst fluttering increased so much that only a semblance of a burst cycle remained. The cycle returned to normal after about five minutes.

As Figure 6B shows, *7%* oxygen completely obliterated the burst cycle. Within one minute the valve opened widely without fluttering, remained open for five minutes with only an occasional closure, and then closed partially five to ten times each minute. Manifestly, this gradual return of spiracular fluttering looks like adaptation, but in our opinion it is more likely the result of a lowered P_{CO_2} within the insect as a result of prolonged spiracular opening (*cf.* Discussion). Within one minute of return to air the valve began to flutter closed and soon thereafter the burst cycle reappeared.

FIGURE 5. Effects of spiracular occlusion on the behavior of the second right abdominal spiracle of a Polyphemus pupa. Air at 25° C. (A) *No* spiracles sealed; 14 functional. A burst occurred just before the recording was begun. For 63 minutes thereafter no bursts occurred. (B) *Ten* spiracles sealed: first, third, fourth, fifth and sixth pairs of abdominal spiracles; four functional. Burst frequency increased from less than one per hour to one every 8 or 10 minutes. The pattern persisted essentially unchanged for 20 hours. (C) *Twelve* spiracles sealed: all of above plus both thoracic spiracles; two functional. Fluttering of valve in open position for periods of 8 to 20 minutes punctuated by brief one-half- to two-minute periods of normal fluttering about the closed position. (D) *Thirteen* spiracles sealed: all of above plus left second abdominal spiracle; one functional. Only the exposed spiracle was available for gas exchange. The result was intermittent closures from a wide-open position, first rapidly and then more slowly.


A
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AIR
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50
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70
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130
C
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FIGURE 6.

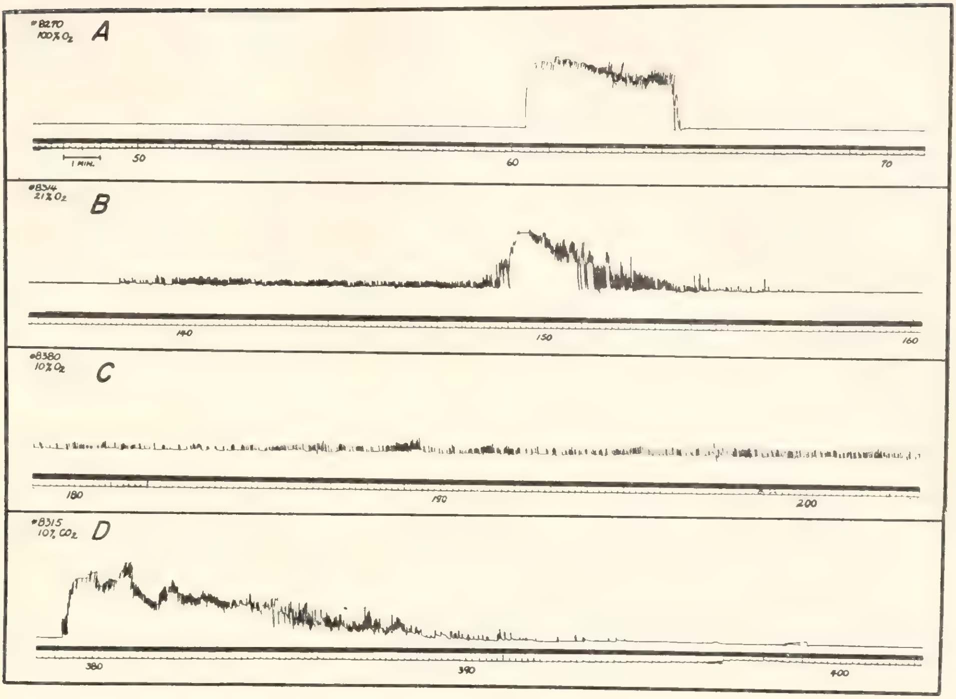

FIGURE 7. Records of spiracular movements of a Cecropia pupa in various ambient gas tensions. In all cases records were made when spiracular activity had reached a steady state. (A) 100% O_2; (B) air; (C) 10% O_2; (D) 10% CO_2. For details see text.

Figure 6C reveals that within 30 seconds of exposure to 5% oxygen, the valve opened widely with no initial fluttering. It gaped open for 70 minutes, closing at irregular intervals for about 40 minutes, and finally stayed wide open and motionless. Within one minute of return to air, fluttering began and continued for 40 minutes, whereupon the burst cycle reasserted itself. Recovery from 5% oxygen thus took nearly forty times longer than recovery from 7% oxygen, suggesting that this low oxygen tension was inadequate for respiration and caused the pupa to become anoxic and build up an oxygen debt. In *pure nitrogen*, a record similar to the 5% oxygen record was obtained (not illustrated). The spiracle closed occa-

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FIGURE 6. Effects of P_{O_2} on the behavior of the second right abdominal spiracle of a diapausing Polyphemus pupa. Three records were obtained in studying each gas mixture: in air, in the specfic gas mixture, and again in air, to appraise the recovery of the spiracle. The time required to change the atmosphere in the chamber was less than 15 seconds. In this experiment and in succeeding ones, the reaction time of the spiracle depended very much on the phase of the burst cycle in which the gas was administered. As one would expect, the most rapid responses occurred when gases were admitted during a spiracular burst. In experiments illustrated the gases were added midway in the interburst period. (A) *15% O_2*. Record of valve movements in air, in *15% O_2 + 85% N_2*, and in air. (B) *7% O_2*. Record of valve movements in air, in *7% O_2 + 93% N_2*, and in air. (C) *5% O_2*. Record of valve movements in air, in *5% O_2 + 93% N_2*, and in air.

sionally for about 10 minutes, but then gaped widely, closing only once during thirty minutes of observation. From these results it appears that decreased oxygen tension can cause valve opening.

A clearer picture of the effects of oxygen tension is seen in Table III, which records the effects of several oxygen tensions on the spiracular behavior of two pupae with longer burst cycles than the pupa just described. These pupae took somewhat longer to respond to both high and low oxygen tensions, often more than 10 minutes except when the gas was administered during a spiracular burst. Figure 7 illustrates portions of several representative records. In *5% to 10% oxygen* the cycles were absent and the valves were continuously in motion. The openings were larger than normal interburst openings, but smaller than normal burst openings. The frequency of flutters, however, was not very different from typical interburst flutters and remained irregular. The period between volleys, however, was much decreased and rarely exceeded 20 seconds, whereas in air, two-minute intervals between volleys of flutters were common.

In *15% oxygen* the bursts reappeared. As the oxygen concentration increased above 15%, the width of valve openings in the interburst decreased, while the frequency remained essentially the same. Also, as oxygen tension increased, the fluttering period got progressively shorter and the constriction period progressively longer. Finally, in pure oxygen all the fluttering before and after the spiracular burst was completely eliminated: the valve remained closed until it abruptly shot open in the burst, and then almost as abruptly closed until the next burst. During the spiracular burst itself, at oxygen tensions above 15%, the higher the oxygen tension, the wider was the valve opening. From these results it appears that oxygen tension affects primarily interburst fluttering but also has some minor effects on the burst itself. It is also noteworthy that increasing or decreasing oxygen tension failed to have any predictable effect on burst frequencies: sometimes frequencies were lowered, other times increased.

TABLE IV

*Effects of P_{CO_2} on various phases of the spiracular burst cycle of a diapausing Cecropia pupa (No. S-83)**

Expt. No.	Ambient P_{CO_2}	Average cycle length (minutes)	Average duration of various phases (minutes)**					Percentage change in spiracular burst	Open	Decline
			Constriction	Percentage change in constriction period	Flutter	Percentage change in flutter period	Spiracular burst			
8311	Air	34	10 (29)	+30	21 (62)	−5	3.1 (9.2)	+62	1.4 (4.1)	1.7 (5.1)
	5%	38	13 (36)		20 (50)		5.0 (13.7)		1.8 (5.1)	3.2 (8.6)
8314	Air	50	16 (32)	+13	29.5 (59)	−81	4.5 (9.0)	+67	1.5 (3.0)	3.0 (6.0)
	8%	31	18 (59)		5.5 (18)		7.5 (24.0)		3.0 (9.7)	4.5 (14.3)
8315	Air	65	12 (19)	0	49 (75)	−91	4 (6.2)	+150	1.0 (1.6)	3.0 (4.7)
	10%	26	12 (45)		4.5 (17)		10 (38)		4.0 (15.0)	6.0 (22.5)
8316	Air	74	10 (14)		61 (81)		3 (4.1)		1.0 (1.4)	2.0 (2.7)
	15%	Continuous flutter	—	—	—	—	—	—	—	—

* Experiments were performed on successive days and the normal and experimental records for each day are paired. Most of the results represent the average of two cycles.

** Figures in parentheses record the *average percentage of the cycle length* occupied by the particular phase.

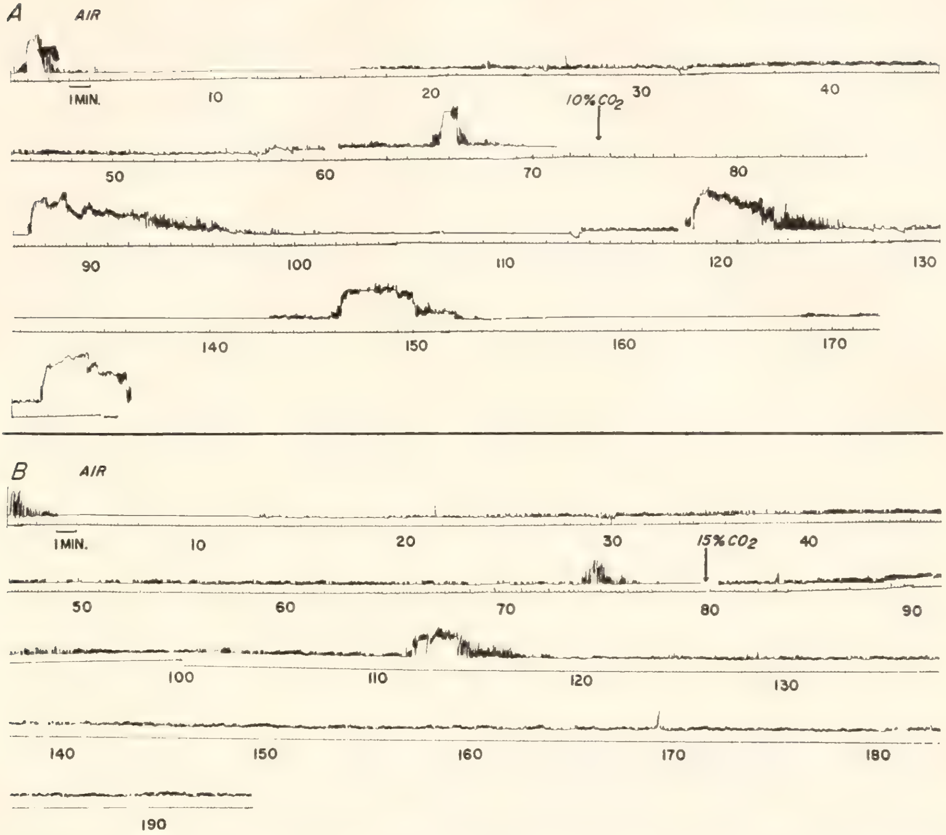

FIGURE 8. Records of spiracular movements of a Cecropia pupa with a moderately long burst cycle in air, followed by 10% or 15% CO_2. (A) 10% CO_2; (B) 15% CO_2.

In occasional experiments, particularly with pupae with high metabolic rates and frequent bursts, pure oxygen failed to suppress interburst fluttering. Instead the spiracular bursts disappeared and the spiracles fluttered continuously. Possible reasons for this curious behavior will be offered in the Discussion.

5. *Effects of carbon dioxide tension*

Increasing the P_{CO_2} increased burst frequency and lengthened both "open" and "decline" phases of the cycle (Table IV and Fig. 8). Besides the effect on burst frequency, the table and figure reveal that high tensions of carbon dioxide also lengthened both the open phase of the spiracular burst and the decline phase after the burst. Thus, in 10% carbon dioxide, the open phases were nearly three times as long as in air (compare Figure 7D with 7B). In addition, although increasing P_{CO_2} had little effect on the duration of the constriction period, it greatly shortened the flutter period until, in 10% carbon dioxide, fluttering was reduced to only a few minutes (Fig. 8). Between 10 and 15% carbon dioxide the burst cycle broke down completely, so that in 15% carbon dioxide the valves fluttered continuously (Fig. 8), and after an initial spiracular burst, no further bursts occurred. The openings were a bit larger than normal interburst flutterings, but much smaller than normal openings in a burst.

The reaction times for a carbon dioxide response never appeared as long as the reaction times for a response to oxygen. Also, pupae with high metabolic rates, and hence frequent bursts, appeared far more sensitive to carbon dioxide than pupae with low metabolic rates. Thus, in a pupa which had a 4- to 5-minute burst cycle, as little as 2% carbon dioxide had a detectable effect on spiracular behavior (prolonged the burst), while 3% eliminated spiracular bursts and provoked continuous wide flutters. Furthermore, when the carbon dioxide tension was raised to 15% in a pupa with such a brief cycle, the valves opened widely, and nearly a half hour was required for recovery upon return to air (Fig. 9). Such sensitivity to carbon dioxide contrasts markedly with the more modest responses recorded in Figure 8, shown by a pupa with a longer burst cycle. A further observation important to our analysis is that in a pupa in which 3% carbon dioxide eliminated the spiracular bursts, bursts could be restored in 3% CO_2 by raising the oxygen tension to about 90%. This observation suggests that high oxygen tensions decrease the sensitivity of the spiracle to carbon dioxide, a fact to which we shall return.

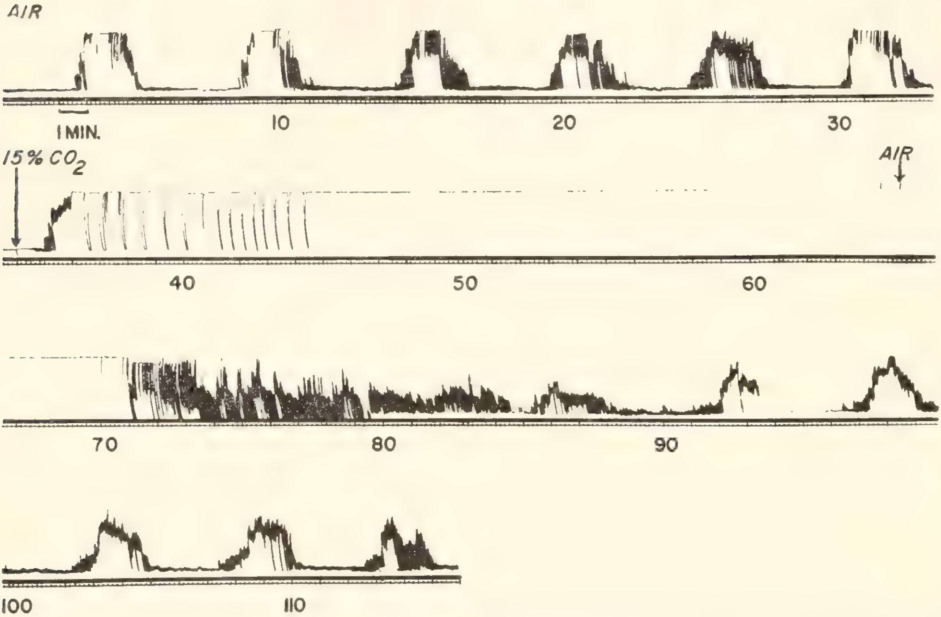

FIGURE 9. Records of spiracular movements of a Polyphemus pupa with frequent bursts in air, in 15% $CO_2 + 21\%$ O_2, and in air.

Although these results convey a general picture of the effects of carbon dioxide and oxygen on the pupal spiracles, they suffer from a conspicuous defect: they provide only little information about the actual gas concentrations *within* the tracheal system that produce a particular effect. In other words, when the insect is exposed to 15% oxygen or 10% carbon dioxide what are the actual intratracheal oxygen and carbon dioxide tensions? A partial solution to this problem was provided by experiments with intubated pupae which are described below.

B. Experiments With Intubated Pupae

1. *Effects of spiracular intubation*

Just as spiracular occlusion decreases the P_{O_2} and increases the P_{CO_2} within the pupal tracheal system, so spiracular intubation has the opposite effect. In the following experiment, the spiracular valves were exposed in four or five abdominal spiracles on one side of a series of diapausing Cecropia pupae. Into two, three or four of these spiracles short fire-polished glass tubes, about 1 mm. in diameter, were inserted beyond the spiracular valves, placing the tracheal system in free communion with the air. This maneuver caused the P_{CO_2} within the insect to fall and the P_{O_2} to rise, as the gaseous composition of the tracheal system approached that of the outside.

One or two days after the operation, and periodically thereafter for about a week, the unintubated spiracles were examined (usually the second and third abdominal). In every case the spiracular valves appeared to stay tightly constricted for many days. They could be opened, however, by exposing the insect to low oxygen or high carbon dioxide. Thus, although the untouched spiracles of the intubated pupa could still function, their cyclical activity had disappeared, presumably because the normal triggering stimuli for spiracular activity were absent.

When intubations were performed on animals with very high metabolic rates (*e.g.* developing adults), despite four intubated spiracles some spiracular fluttering occurred. Apparently, intubating even four spiracles in animals with high metabolic rates does not permit equilibration of internal and external gases.

These experiments, coupled with the other experiments considered thus far, are consistent with the view that the triggering stimulus for spiracular opening is either low internal tension of oxygen, high internal tension of carbon dioxide, or a combination of the two. This matter is considered in further detail below.

2. *Effects of oxygen tension on the spiracles of intubated animals*

Intubation eliminates the spiracular burst cycle and the spiracles exhibit a persistent pattern of behavior in various gas mixtures. Thus, the intubated pupae provide a simple means of determining the approximate concentrations of oxygen and carbon dioxide that produce a particular kind of spiracular behavior, *e.g.* valve fluttering, bursts etc., and also a means of examining the interaction between carbon dioxide and oxygen in controlling spiracular movement. To conduct these experiments, pupae with two or three intubated spiracles were exposed to various gas mixtures in a flow system, and the movements of two normal spiracles observed until they exhibited a constant behavior. It took anywhere from 5 to 35 minutes for the constant response to assert itself, except in very low oxygen tensions or very high carbon dioxide tensions where the response appeared more rapidly. The decision to study the final response of the spiracles rather than their initial response was taken for several reasons. The principal one was that, in these large insects, it very likely takes many minutes for gases to reach a steady-state. A second

TABLE V

*Determination of the "O_2-open" threshold of two spiracles of an intubated Cynthia pupa**

Per cent oxygen	Response of spiracle 2R	Response of spiracle 3R
21	Closed	Closed
15.5	Active fluttering	Closed
8.5	Partly open with rapid closures	Slight fluttering (pulsation)
5.0	Wide open with occasional flutters	Active fluttering
4.5	Wide open with occasional flutters	Active fluttering
4.0	Wide open with occasional flutters	Wide open with occasional flutters
3.5	Wide open with occasional flutters	Wide open with occasional flutters
3.0	*Wide open with no closures*	Wide open with occasional flutters
2.5	*Wide open with no closures*	Wide open with occasional flutters
2.0	*Wide open with no closures*	*Wide open with no closures*
1.5	*Wide open with no closures*	*Wide open with no closures*

* Almost identical results were obtained when the gas mixtures were flushed through the chamber at random instead of in order, provided adequate recovery periods were allowed. Hence, under the conditions of our experiments, previous exposure to a test gas mixture had no effect on subsequent tests.

reason was that, in the normal intact insect, gas changes are ordinarily gradual and not cataclysmic, and hence it seemed a sensible procedure to allow time for adjustment to each change of gas.

Table V illustrates the data recorded to determine the effects of oxygen on two spiracles of a typical diapausing pupa. Progressive lowering of the ambient oxygen tension caused in sequence: pulsation of the valve (no visible opening), fluttering (small openings), partial openings and closings, full opening and occasional closure, and finally full opening with no movement, *i.e.*, complete relaxation of the spiracular closer muscle.

TABLE VI

"O_2-flutter" thresholds and "O_2-open" thresholds of abdominal spiracles of five intubated Cecropia pupae

Experiment No.	Spiracle observed	"O_2-flutter" threshold* (per cent)	"O_2-open" threshold** (per cent)
21 b	1 L	12	3
	3 L	8.5	3
22 b	1 L	12	1.5
	2 L	12	1.5
23 b	2 L	8.5	1.5
	3 L	5.5	1.5
24 b	1 L	12	1.5
	2 L	8.5	1.5
25 b	2 L	5.5	1.5
	3 L	5.5	1.5

* P_{O_2} which caused first pulsations.

** P_{O_2} which caused full spiracular opening for one minute.

Two oxygen tensions are of special interest: the tension that initiates spiracular movement, *i.e.,* pulsations or fluttering, and the tension that causes a spiracle to open fully, as in a burst. A typical set of values for these two oxygen tensions for a series of diapausing pupae is recorded in Table VI. From the table it can be seen that the five pupae studied varied considerably in their sensitivity to oxygen. Spiracular movements began at oxygen tensions ranging from 12 to 5.5%. The tensions required for full opening were more uniform. Unfortunately, the oxygen tension that induced pulsations proved to be exceedingly difficult to determine since it was not reproducible in successive experiments. The oxygen tension that produced full opening of the spiracle was far simpler to ascertain. Therefore, to facilitate quantitative measurements we concentrated on one spiracular response: full opening of the valve for one minute. This response occurs normally during a burst cycle, was easily judged, and was altered by changes in oxygen or carbon dioxide tensions of ± 0.5%. Moreover, it was easily reproducible.

The tension of oxygen necessary to open the spiracles widely was determined for 33 spiracles of more than a dozen Cynthia pupae that had several spiracles intubated. This tension of oxygen, hereafter termed the "O_2-open threshold," averaged 2.58 ± 1.12(s.d.)% with a range of 1% to 5.5%. Two-thirds of the spiracles responded to between 2% and 3.5% oxygen. Within any pupa the O_2-open threshold for

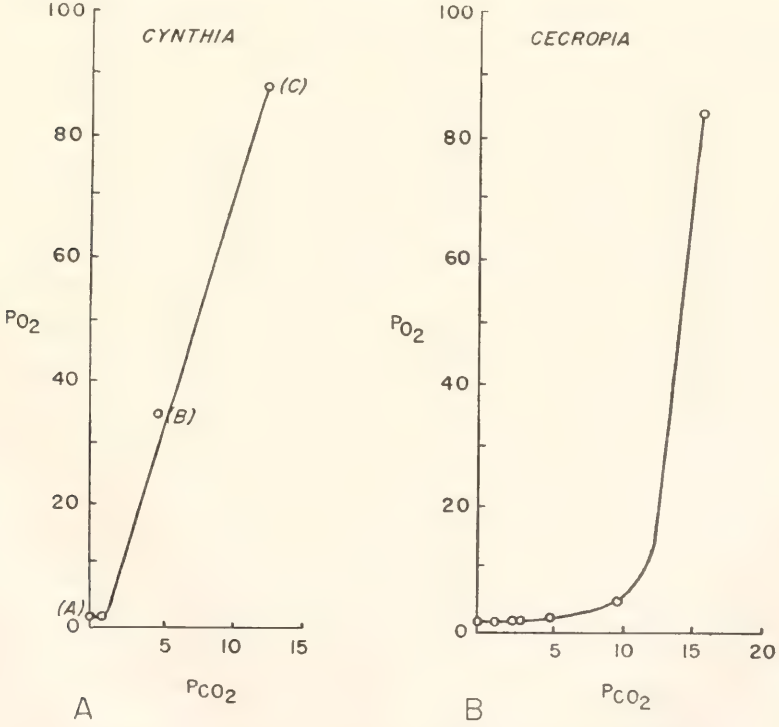

FIGURE 10. (A) The effect of P_{O_2} on the P_{CO_2} necessary to open a spiracle of an intubated Cynthia pupa widely for one minute. Partial pressures are expressed as % atm. For details see text. (B) Same curve as above for an intubated Cecropia pupa.

different spiracles did not vary by more than 1.5%. For a given spiracle, at all oxygen tensions below threshold the spiracle was wide open and the spiracular muscle remained relaxed; at tensions 0.5% or more above the O_2-open threshold, intermittent to prolonged contractions of the muscle occurred, which closed the valve (*cf.* Table V). The O_2-open threshold was also determined for a few developing adults of Polyphemus and found to average 3.1% oxygen with a range of 2.0–4.5%.

3. *The interaction between oxygen and carbon dioxide in the spiracles of intubated animals*

The carbon dioxide tension necessary to open the spiracle widely for one minute varied with the oxygen tension. This is seen in Figure 10A which records the behavior of a Cynthia pupa. The first point (A) on the curve gives the highest partial pressure of oxygen in a nitrogen-oxygen mixture that kept the spiracle open, *i.e.*, the O_2-open threshold which we considered in the previous section. The third point (B) on the curve was determined by adding a mixture of 4% carbon dioxide to air, a mixture which kept the spiracle wide open, and progressively increasing the oxygen tension until the spiracle began to close. In this experiment, in the presence of 4% carbon dioxide, oxygen tensions above 35% were necessary to close the spiracle. In other words at any point along the curve for a given partial pressure of carbon dioxide there was a critical tension of oxygen, below which the valve would remain fully open and motionless; oxygen tensions above this critical tension caused closure of the valve. The end-point of this curve is (C). At carbon dioxide tensions above this value the valve remains open and motionless and no partial pressure of oxygen at atmospheric pressure will cause fluttering. This point, which is designated the "CO_2-open threshold," was determined by starting in pure oxygen where the valve was closed and increasing the carbon dioxide tension until a tension was reached at which the spiracle remained open for one minute. This CO_2-open threshold was simple to determine and reproducible within about 1%. Several curves of this sort were recorded for a number of pupae and all looked essentially the same. One typical of a Cecropia pupa is presented in Figure 10B.

Although such curves are difficult to obtain, by measuring only the O_2-open threshold and the CO_2-open threshold of a spiracle it was possible to assess in a general way the sensitivity of the spiracle and to compare spiracles of pupae under various conditions. This was done for a series of Cynthia spiracles and the O_2-open thresholds have already been discussed. The CO_2-open thresholds of this same series of spiracles averaged 17.63 ± 5.42(s.d.)% and covered a far greater range (13.5% to 31%) than the O_2-open thresholds. Notwithstanding, two-thirds of the spiracles had a CO_2-open threshold between 14% and 16%. The CO_2-open threshold appeared quite uniform for different spiracles of any individual. A few determinations were also made on the spiracles of developing adults of Polyphemus. Whereas the O_2-open thresholds for these insects with high metabolic rates did not differ significantly from the thresholds for diapausing pupae, the CO_2-open thresholds of the developing adults were lower than for the diapausing pupae and averaged only 10.5% with a range of 9.5–12.0%. Furthermore, the CO_2-open thresholds decreased on successive days of adult development as the metabolic rate increased.

These results demonstrate the influence of oxygen on the response of the spiracle to carbon dioxide, and indicate that the sensitivity to carbon dioxide decreases as oxygen tension increases. Similar results were obtained when the carbon dioxide tension that caused fluttering was determined: the sensitivity to carbon dioxide decreased as the oxygen tension increased.

Discussion

1. *Scope of the analysis*

The spiracular behavior of giant silkworms just described represents one of the most extreme kinds of spiracular behavior recorded for any insect, with a complex cycle of activity which commonly lasts for hours and occasionally for days. The present experiments confirm and amplify earlier opinions that the cyclical behavior of the spiracles is somehow responsible for discontinuous respiration. They may have, in addition, some intrinsic interest for insect respiration as a whole. For, in a sense, these giant pupae breathe in "slow motion," and events which may occupy only a second or two in the respiratory cycles of other insects may take many minutes or even hours. This enables the experimenter to analyze complex events in gas exchange and spiracular behavior which are not readily separable in insects with brief cycles. The present experiments have exploited this property in an effort to define the kinds of behavior that spiracles can show, and to clarify the manner in which oxygen and carbon dioxide interact to provoke various modes of spiracular activity.

2. *The behavior of the spiracular muscle*

Interpretation of the present experiments is simplified by the fact that the movements of each pupal spiracle are controlled by a single muscle. Thus, the records of spiracular activity are records of the behavior of this muscle. As we have seen, the muscle displays three sorts of behavior: sustained contraction which causes valve closure; brief partial relaxation which causes valve fluttering; and almost complete relaxation which causes the valve to gape open.

In the normal pupa these different kinds of behavior occur in two characteristic patterns: (1) the first is the "flutter," in which the muscle relaxes slightly and then quickly re-contracts fully. This occurs many times each minute in irregular volleys and may continue for hours. It is important to note that the relaxations are very brief and that during most of the flutter period the valves are constricted. Indeed, as far as one can tell, they are as tightly constricted between flutters as they are during the constriction period after a burst. (2) The second pattern, the "burst" cycle, is more dramatic and far more regular. It is compounded of numerous flutters and periods of prolonged valve opening and prolonged constriction.

The initiation of each of these patterns of spiracular behavior does not appear to depend upon an endogenous nervous rhythm as is true, for example, in the respiratory movements of *Schistocerca* and *Dixippus* (*cf.* review by Wigglesworth, 1953a, and also Hoyle, 1959) or of vertebrates, but is the result of cyclical changes in the composition of the tracheal gases. The mechanism of this extrinsic control is described below.

3. *An explanation for the burst cycle*

All events in the normal burst cycle, as well as the experimental observations, can be explained in terms of the following three properties of the spiracular muscle and its associated nerves: (a) the muscle is ordinarily continuously stimulated to contract; (b) when tracheal P_{O_2} falls below a critical value, the flutter threshold, the muscle repeatedly relaxes slightly and re-contracts; (c) when tracheal P_{CO_2} rises above a critical value, the burst threshold, the muscle relaxes fully. These assertions permit us to frame the following hypothesis which appears to be consistent with all of our observations and accounts for the burst cycle.

Immediately after a burst the valves are constricted, tracheal P_{O_2} is far above the flutter threshold, and tracheal P_{CO_2} is far below the burst threshold. This is the constriction period. As the insect respires, oxygen inflow fails to match oxygen utilization and the P_{O_2} within the tracheal system falls. When the tracheal P_{O_2} falls to about 3%, the flutter threshold, spiracular fluttering begins, permitting oxygen to enter. This increases the tracheal P_{O_2} and the valve promptly closes. This pattern of rapid fluttering, punctuated by short periods of constriction, represents the flutter period. In high oxygen tensions tracheal P_{O_2} never reaches triggering concentration and hence no fluttering occurs.

What about the spiracular burst itself? This involves carbon dioxide. During the interburst period some carbon dioxide escapes, but at a rate considerably less than that of its production. As a consequence, internal P_{CO_2} ultimately reaches the spiracular burst threshold, and the spiracular muscle relaxes. The valve opens widely in a burst; carbon dioxide and nitrogen flow out and oxygen flows in, as the composition of the tracheal gas approaches that of the atmosphere. When the internal P_{CO_2} decreases below a certain value, the valve begins to open and close periodically, with diminishing width and duration of opening, until it finally remains constricted and the cyle is completed.

Detailed support for this hypothesis and for the assertions regarding the effect of P_{O_2} and P_{CO_2} on the spiracular muscle are examined below.

4. *Oxygen effects: the constriction and flutter periods*

A number of experiments prove that fluttering is caused by low tracheal P_{O_2} and is little affected by tracheal P_{CO_2}. Firstly, in a normal pupa, lowering ambient P_{O_2} to about 10% evokes continuous valve fluttering (presumably because tracheal P_{O_2} is lowered). Secondly, raising ambient P_{O_2} suppresses entirely the valve fluttering of a normal pupa: apparently under this condition tracheal P_{O_2} never falls to the flutter threshold. Thirdly, when several spiracles are intubated, thereby raising the internal P_{O_2}, fluttering ceases. Finally, in such an intubated pupa, when ambient P_{O_2} is lowered to about 2% fluttering continues indefinitely.

Under this analysis, in a normal pupa the *duration of the constriction period* after a burst is determined primarily by the rate at which P_{O_2} falls within the tracheal system. This in turn depends upon three factors: (1) the tracheal volume of the pupa, (2) the rate of oxygen utilization by the pupa, and (3) the rate at which oxygen leaks in through the constricted spiracles. In general, large pupae with low metabolic rates have long constriction periods. Also, as one would predict, the constriction period is prolonged by increasing ambient P_{O_2} (*cf.* Table III), for at the end of a burst, not only is there a higher O_2 content in the tracheal system, but also more O_2 leaks inward.

In occasional experiments, pure oxygen failed to suppress valve fluttering but instead abolished the burst cycle. Similar results were found by Buck *et al.* (1955) in manometric experiments with *Agapema* pupae. Neuromuscular mechanisms of insects are particularly susceptible to oxygen poisoning (Goldsmith and Schneiderman, 1960) and this may account for these results.

What determines the frequency of fluttering and the duration of the closed period between each volley of flutters? Both of these appear to be governed, as is the duration of the constriction period, by the rate at which P_{O_2} falls within the tracheal system, and therefore depend in part upon metabolic rate and upon ambient P_{O_2}. As one would expect, the higher the metabolic rate, the more frequent the flutters and the more frequent the volleys of flutters (*cf.* Figures 4 and 6). Moreover, since the frequency of the flutters and the frequency of volleys depend primarily upon tracheal P_{O_2}, it is not surprising that they do not change systematically during the course of the flutter period, for *once fluttering begins, tracheal P_{O_2} presumably varies very little since oxygen continuously enters.* This prediction has been confirmed by analyses of tracheal gas composition (Levy and Schneiderman, 1957, 1958), which will be discussed in detail in a subsequent report. For our present purposes this constant P_{O_2} is of singular importance, for it tells us that the termination of the flutter period by a spiracular burst depends not upon a change in tracheal P_{O_2}, but upon the accumulation of carbon dioxide. This is considered below.

5. *Carbon dioxide effects: "triggering" and termination of the spiracular burst*

The most conspicuous effect of an increase in ambient P_{CO_2} was the shortening of the burst cycle, which confirms previous manometric findings (Schneiderman and Williams, 1955). This reduction in cycle length was largely at the expense of the flutter period, which was greatly shortened, whereas the duration of the constriction period was unchanged. In short, increasing ambient P_{CO_2} led to the premature triggering of the burst early in the flutter period. Moreover, once triggered, the burst was prolonged. These observations can be understood in the following terms:

(1) *The absence of an effect of carbon dioxide on the duration of the constriction period* supports the opinion offered above, that in the intact pupa the onset of fluttering is triggered primarily by low tracheal P_{O_2}. Further support comes from the fact that the P_{O_2} which triggered off the fluttering was not significantly affected by modest increases in tracheal P_{CO_2}. Indeed, in pupae with long cycles, until ambient P_{CO_2} rose above 10%, it did not appear to affect the amplitude or frequency of flutters.

(2) *The premature triggering of spiracular bursts by ambient P_{CO_2}.* A variety of experiments revealed that in a normal burst cycle, the spiracular bursts themselves are triggered by tracheal P_{CO_2}, and not by tracheal P_{O_2}. Thus exposing both normal and intubated pupae to physiological and non-anaesthetic concentrations of carbon dioxide causes the spiracular valves to open widely. In a normal cycle, tracheal P_{CO_2} gradually increases after a burst as a result of metabolism and, when it reaches a certain level, the spiracular muscle fully relaxes and a burst occurs. At *all* concentrations of carbon dioxide, tracheal P_{CO_2} more quickly reaches the

triggering concentration, because the partial pressure gradient driving carbon dioxide out of the insect is decreased, with a consequent decrease in the rate of carbon dioxide leakage. In addition, *high* ambient concentrations of carbon dioxide (*i.e.*, above 5%) prevent tracheal carbon dioxide from falling to its normal low level after a burst. This constant high level of tracheal carbon dioxide also shortens the time required to reach a trigger value. Thus, for these two reasons, the higher the ambient carbon dioxide concentration, the shorter the time between bursts. The second reason is by far the more important in pupae with low metabolic rates. In such insects ambient carbon dioxide tensions below 5% had no detectable effect on burst frequency, whereas increasing carbon dioxide from 5% to 10% often increased the burst frequency 10 to 20 times (Schneiderman and Williams, 1955). However, in pupae with high metabolic rates and high burst frequencies, the reduction in carbon dioxide escape caused by the presence of even 1% ambient carbon dioxide was often sufficient to abolish the bursts entirely.

A different kind of evidence for carbon dioxide triggering spiracular bursts comes from experiments on *Agapema* pupae, in which there was demonstrated an inverse relation between ambient P_{CO_2} and the *delay* in the first subsequent spiracular opening (Buck and Keister, 1958). Pupae in 9% carbon dioxide opened their spiracles 2¾ hours after exposure, whereas in 24% carbon dioxide they took only one hour. As the authors point out, this is what would be expected "if the carbon dioxide were leaking into the tracheal system and accelerating the rise to triggering concentration" (p. 335).

(3) *Further evidence for the triggering of spiracular bursts by tracheal* P_{CO_2}. The triggering of bursts by tracheal P_{CO_2} is well seen in 100% oxygen, where internal P_{O_2} might be expected to remain relatively high throughout the cycle. Here it seems clear that increasing tracheal P_{CO_2} was the trigger that caused the spiracular muscle to relax. The converse result is seen in intubated pupae, where one prevents carbon dioxide from reaching a concentration high enough to trigger a spiracular burst and the valves remain permanently closed.

(4) *Termination of the spiracular burst.* Once triggered to open in a burst, the spiracle remains open and carbon dioxide diffuses out until tracheal P_{CO_2} falls to some critical level whereupon the spiracle closes again. Increased ambient P_{CO_2} prolonged bursts, presumably because it delayed the diffusive outflow of carbon dioxide. It is worth recalling that it takes the blood and tissues some time to unload carbon dioxide. In most cases, long before this is done the tracheal P_{O_2} has risen to its maximum value as oxygen diffuses in at the burst. Hence, the closing of the spiracle at the end of a burst has much less to do with tracheal P_{O_2} than with tracheal P_{CO_2}.

It is puzzling that immediately the carbon dioxide tension falls somewhat, the muscle does not contract again, but instead remains relaxed for many minutes, even when carbon dioxide tension has fallen well below the triggering threshold. In some way, high P_{CO_2} provokes a response in the muscle which is sustained for a considerable period (*cf.* discussion of this point by Buck, 1957). This sharply contrasts with the partial relaxations induced by low P_{O_2}, which are very transitory, and suggests that flutters and spiracular bursts are fundamentally different responses.

6. *Effects of metabolic rate*

The effects of metabolic rate on spiracular activity appear to be similar in most insects: high metabolism leads to increased spiracular movement (*cf.* review by Wigglesworth, 1953a). For example, Nunome (1944) has reported that the spiracles of *Bombyx mori* open more frequently and more widely when larvae were active than when they were inactive. In the present experiments, spiracular burst frequency increased with metabolic rate, confirming manometric studies. This increase in frequency is largely the result of more rapid production of carbon dioxide which shortens the time required for tracheal P_{CO_2} to reach triggering concentration. The more rapid decrease in tracheal P_{O_2} which attends high metabolism probably has only a limited impact, inasmuch as once fluttering begins, tracheal P_{O_2} remains constant.

When metabolic rate gets very high and bursts occur every 5 or 6 minutes (see for example Figure 6), the constriction period is obliterated, and between bursts the valves are in continuous motion, *i.e.,* fluttering. In effect, the decline period after the burst fuses with the flutter period before the next burst. This suggests that oxygen utilization is so rapid that even during the decline period tracheal P_{O_2} falls rapidly to the flutter threshold. When metabolic rate gets very high and the cycles last for less than four minutes, as is the case with late developing adults, the interburst flutterings increase markedly in amplitude until the burst cycles disappear entirely and are replaced by continuous wide flutters. As mentioned previously, these flutters are far larger than oxygen-induced flutters. It appears likely that when metabolism gets high enough to cause continuous fluttering, carbon dioxide may be viewed as the principal stimulus. Further support for this opinion comes from the observation that in intubated pupae the O_2-open threshold did not vary with metabolic rate, whereas the CO_2-open threshold decreased as metabolic rate increased. This suggests that in pupae with high metabolic rates, there is a considerable accumulation of carbon dioxide even when several spiracles are open. This high tracheal P_{CO_2} probably accounts for the fact that intubated pupae with very high metabolic rates showed continuous valve fluttering.

7. *Interaction between carbon dioxide and oxygen*

In the above analysis we have considered oxygen and carbon dioxide as acting essentially independently. This is not the case, but, as we shall see, this fact does not impair the analysis. Evidence that oxygen tension affects the carbon dioxide threshold was provided by the observation that high oxygen tension markedly reduced the burst frequency from 5 to 10 bursts per hour to one to two per hour in a pupa in which 5 pairs of spiracles were sealed. This finding suggests that the high oxygen tension raised the carbon dioxide threshold so that more carbon dioxide had to accumulate to cause a spiracular burst. A further piece of circumstantial evidence was the discovery that very high oxygen tensions often restored bursts in pupae in which carbon dioxide had eliminated the bursts. However, interpretation of these experiments was hampered by the fact that it was difficult to be certain of the tracheal gas composition, which varied continuously. Part of this difficulty was by-passed in experiments with intubated pupae. To be sure, even in intubated pupae the tracheal gas composition is not known with certainty, but at least it can be kept constant and the oxygen and carbon dioxide tensions can be varied independently.

The intubation experiments reveal that the concentration of carbon dioxide necessary to open the spiracles wide varies with the oxygen tension. In the presence of 2.3% oxygen, 5% carbon dioxide opened the spiracles of a typical Cecropia pupa, whereas in 4.7% oxygen, 10% carbon dioxide was required. This result demonstrates clearly the interaction between P_{O_2} and P_{CO_2} in controlling the wide opening of spiracles, and it finds several parallels in the literature. Thus, in the cockroach (Hazelhoff, 1926a, 1926b), the flea (Wigglesworth, 1935), and in various muscid flies (Case, 1956a), the opening of spiracles in response to carbon dioxide is favored by low oxygen and depressed by high oxygen, just as in Cynthia and Cecropia pupae.

In the present experiments it is of some interest that, although P_{O_2} influenced the P_{CO_2} necessary to cause a spiracular burst, there did not seem to be a similar interaction in the triggering of flutters. Thus, as discussed in Section 5 above, increasing ambient P_{CO_2} up to 10% had no significant effects on the oxygen flutter threshold. Hence, it seems unlikely that during a normal burst cycle internal P_{CO_2} affects the oxygen flutter threshold, inasmuch as tracheal P_{CO_2} in these pupae rarely rises above 6.5% (Buck and Keister, 1958; Levy and Schneiderman, 1958).

It is not our present purpose to explore the mechanism of the interaction between oxygen and carbon dioxide in triggering bursts. However, recognition of this phenomenon enables us to interpret several events in the burst cycle to which we have not yet attended, notably the onset of the decline period. Why does the relaxed spiracular muscle start contracting again about one-third of the way through a burst? This might be the result of falling tracheal P_{CO_2}, rising tracheal P_{O_2}, or both. Evidence that it is largely rising P_{O_2} comes from records of pupae in pure oxygen. Here there is no decline period and the spiracle simply shuts. This suggests that under normal conditions the decline period is related to tracheal P_{O_2}. A possible mechanism might be the following.

During a spiracular burst, tracheal P_{O_2} equilibrates with the atmosphere far more rapidly than does tracheal P_{CO_2} because of the long time required to unload dissolved carbon dioxide. In a relatively short time, tracheal P_{O_2} rises to a concentration that increases the carbon dioxide burst threshold. The valve shuts briefly, to reopen promptly as carbon dioxide continues to accumulate in the tracheal system. Under this analysis, the onset of the decline period marks the point in a spiracular burst where tracheal P_{O_2} has risen high enough to depress appreciably the sensitivity of the spiracle to carbon dioxide. As mentioned in Section 5 above, the duration of the decline period is determined by the time necessary to unload carbon dioxide.

A second instance of O_2-CO_2 interaction appears to occur in the period just before a spiracular burst. Ordinarily, 20 to 120 seconds before a burst, flutters are larger than normal and gradually build up to the burst. This brief build-up period resembles the decline period and is also absent in pure oxygen, suggesting that, like the decline period, it reflects O_2-CO_2 interaction. The mechanism of this interaction will be considered subsequently, but for the present we can conclude that at the low P_{O_2} which obtains in a normal pupa just prior to a burst, the carbon dioxide threshold of different excitatory elements is not as uniform as in high P_{O_2}.

Interaction between oxygen and carbon dioxide also explains why variations in ambient P_{O_2} have no predictable effect on burst frequency. High ambient P_{O_2} raises the carbon dioxide threshold of the spiracles, and this tends to decrease the burst frequency. But, simultaneously, high oxygen decreases interburst fluttering and this, in turn, decreases the rate of interburst carbon dioxide release, and increases the rate of carbon dioxide accumulation. This tends to increase the burst frequency. The ultimate outcome, as we know from manometric experiments (Schneiderman and Williams, 1955) and from the present study, is sometimes increased frequency, sometimes decreased frequency, and in other cases no change.

Table VII summarizes the conclusions reached in this discussion relating to the effects of tracheal P_{O_2} and P_{CO_2} on the behavior of the spiracular valves during the burst cycle.

TABLE VII

Summary of the effects of tracheal P_{O_2} and P_{CO_2} on the behavior of the spiracular valves during a burst cycle

Tracheal composition	Constriction period		Flutter period		Burst	
	Initiate	Terminate	Initiate	Terminate	Initiate	Terminate
Low P_{CO_2}	+	0	0	0	0	+
High P_{CO_2}	0	(+)	(+)	+	+	0
Low P_{O_2}	0	+	+	(+)	(+)	0
High P_{O_2}	±	0	0	0	0	±

+ Principal agency responsible.
± Secondary factor.
(+) These conditions do not normally prevail in the phase of the burst cycle indicated.

8. *The existence of an independent oxygen-sensitive mechanism*

It is not a principal purpose of the present report to discuss the mechanisms whereby oxygen and carbon dioxide exert their effects, nor to consider the sites of action of these gases. Earlier experiments on these pupae ruled out the existence of any indispensable respiratory center (Schneiderman and Williams, 1953b; Schneiderman and Beckel, 1954; Schneiderman, 1956). They also showed that although nervous control was involved in the normal stimulation and coordination of the spiracular muscles (Schneiderman, 1956), the immediate response to carbon dioxide resided in the spiracular muscle itself (Beckel and Schneiderman, 1956, 1957). However, the present studies appear to clarify one point which has hitherto not been resolved for any insect, namely that, at least in silkworm pupae, *oxygen and carbon dioxide affect the spiracle via very different mechanisms.* It has been suggested by various workers that oxygen-lack produces acidity, and this might be its mode of action in stimulating spiracles. Wigglesworth (1935, 1953b) argues that in the flea both O_2-lack and CO_2-excess act in virtue of the acidity they produce. Case (1957a) and Punt *et al.* (1957), on the other hand, raise the possibility that the acidity produced by oxygen-lack might release bound carbon dioxide from the blood and that CO_2 is the effective agent to which spiracles respond in both O_2-lack and CO_2-excess. In the case of the silkworm, the experimental results forbid either interpretation, as the following considerations show.

During the course of a burst cycle, when P_{O_2} falls to a critical threshold, fluttering begins and may continue uniformly for many hours. This sustained pattern of behavior in response to low P_{O_2} indicates that the primary response to oxygen lack is not acidity itself, nor acidity releasing bound carbon dioxide. For, during the entire course of the flutter period, acidity increases steadily as carbon dioxide accumulates. Notwithstanding, the flutter response did not change appreciably, nor did it change after ambient P_{CO_2} was raised to 10% (*cf.* Figure 8 and Section 5 above), a maneuver which certainly increased acidity. These observations suggest that, in the silkworm, increasing acidity is probably not the means whereby oxygen lack makes itself felt. They also seem to prove that in the silkworm pupa there is a specific mechanism sensitive to oxygen lack that may act independently of any carbon dioxide-sensitive mechanism.[4] Evidence will be presented in a subsequent paper that P_{O_2} may act on the central nervous system in contrast to P_{CO_2} which acts peripherally.

9. *Comparison with the flea*

In concluding, it is fruitful to compare the spiracular behavior of these silkworm pupae with the behavior of the spiracles of the flea described by Wigglesworth (1935) in his fundamental study. The flea spiracle is operated by a single closer muscle and shows a simple pattern of opening and closing. In air at 20 to 22° C. cycles are 12 to 16 seconds long, 6 to 8 seconds during which the spiracle stays closed followed by about an equal time during which the spiracle is open. P_{O_2} and P_{CO_2} affected this cycle in a predictable way. As P_{O_2} diminished from 100% there was an increase in cycle frequency; the closed period was considerably shortened, whereas the open period was less affected. As P_{CO_2} increased, the open period was lengthened considerably, whereas the closed period was slightly shortened. It was concluded that the spiracles were caused to open chiefly by falling P_{O_2}, rising P_{CO_2} being less important.

In the pupal burst cycle, the constriction period corresponds to the closed period in the flea, and the spiracular burst corresponds to the open period in the flea. Because the flea has no flutter period we cannot compare the effects of various gas mixtures on the length of an entire cycle (*i.e.*, on the frequency of the cycles), but we can compare the periods of valve closure and of valve opening in both insects.

(a) *Oxygen effects*

In both the flea and the pupa the duration of the constricted period is proportional to P_{O_2}, and in both, the spiracle is caused to open by low P_{O_2}. However, unlike the flea spiracle, which opens widely and stays open for a number of seconds after a constriction period, the pupal spiracular muscle relaxes only slightly when a critical P_{O_2} is reached and then re-contracts immediately. This behavior is repeated over and over during the flutter period, for which there is no counterpart in the flea. In the pupa, for reasons already discussed in Section 7, P_{O_2} has no predictable effect on cycle frequency.

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[4] Very recently Hoyle (1960) has provided convincing evidence that in the spiracular muscle of the locust, CO_2 acts directly on the neuromuscular junction and blocks transmission of nervous excitation. This important discovery supports the view outlined above that CO_2 acts peripherally.

(b) *Carbon dioxide effects*

In both the flea and the pupa the duration of the spiracular burst or open period is proportional to P_{CO_2}. And in both, P_{CO_2} has only small effects on the duration of the constriction period. It is noteworthy that in the flea, carbon dioxide fails to affect the frequency of openings and in the pupa it fails to affect the frequency of flutters or volleys of flutters in the flutter period. These observations support the view that the open period of the flea and the flutter period of the pupa are largely triggered by low tracheal P_{O_2}, and that tracheal P_{CO_2} is ordinarily of much less importance in this connection. It is nonetheless of some significance that, in the flea, raising the P_{CO_2} decreased the closed period, albeit only slightly. In commenting upon this, Wigglesworth (1935) remarks (p. 405) that the closed period of the flea is terminated "chiefly by oxygen want (carbon dioxide contributing to a small extent)." The pupal spiracular burst cycle enables us to separate clearly the oxygen-want effect from the carbon dioxide effect, for in the pupa the oxygen-want effect—fluttering—not only appears first, but is qualitatively different from the carbon dioxide effect—the initiation of a spiracular burst. In only one place in the flea are these effects separable, namely, in pure oxygen. Here, in the flea as in the pupa, the spiracular bursts seem to have been triggered mainly by P_{CO_2} and not by oxygen lack. It is also noteworthy that in both the flea and the pupa, the effects of carbon dioxide varied directly with the intensity of metabolism.

A further point of similarity between the flea and the pupa is that in both, the denervated spiracular muscle remains contracted (Wigglesworth, 1935; Schneiderman, 1956). In the case of the pupal silkworm the muscle remains contracted for many weeks, and this appears to be true also of the bed-bug (Wigglesworth, 1941) and the roach (Case, 1956b, 1957b). These are all insects without nervous-controlled ventilation movements under ordinary conditions. By contrast, the spiracular muscles of insects like *Schistocerca*, which have ventilation movements, relax when denervated (Fraenkel, 1932; Hoyle, 1959). Indeed, it may be that in many insects where the rhythmical activity of spiracles is under nervous control, denervation leads to relaxation, whereas in others, like the flea and silkworm pupae, where the spiracles are under the extrinsic control of respiratory gases, the denervated spiracular muscle continues to contract.

In sum, it appears that the behavior of the spiracles of both the flea and the pupa is remarkably similar. A crucial difference between the two respiratory cycles is the flutter period of the pupa. This cannot stem simply from differences in the intensity of metabolism, for when fleas are placed at temperatures as low as 4° C., which reduces their metabolism to levels characteristic of diapausing pupae, they do not show flutters. Similarly, raising the metabolism of pupae to that of fleas by injury or by other means fails to produce the flea pattern of prolonged openings. The reason for the different modes of spiracular behavior is probably to be found, at least in part, in the size of the insects and the attendant differences in the lengths of both fluid and gaseous diffusion paths. Contributing also to the differences in spiracular behavior is the relative insensitivity of the O_2- and CO_2-sensitive mechanisms. This was recognized at the outset by Wigglesworth (personal communication), who pointed out that among the preconditions for discontinuous respiration was that the responding system "must have an extremely high threshold of stimulation by carbon dioxide . . . and this means that there must be a very large accumulation of CO_2 before it will cause the spiracles to open." This is surely so, and the CO_2 threshold of the flea is likely much lower than that of the pupa.

However, the differences between the spiracular behavior of the pupa and the flea are modest when weighed against the similarities. In summarizing his findings on the flea, Wigglesworth (1935) concluded (p. 402) that each respiratory act was "determined by an immediate stimulus of a chemical nature." The present experiments extend these conclusions to the silkworm and indicate that carbon dioxide bursts result in large measure from an exaggeration of a basically simple sort of spiracular behavior.

10. *Comparison with other insects*

When the spiracles of other insect groups are examined, it becomes immediately clear that fluttering, although not unique to silkworms, may be less common than the simple closing and opening observed in the flea. For example, Hazelhoff (1926a) reported no fluttering in any of the Diptera, Trichoptera, Neuroptera and Odonata that he examined. However, he says that in *Periplaneta americana* ". . . All the stigmata are almost closed. . . . The movable stigmal valve performs *usually quick vibrating movements*" (flutters) "whereby nevertheless the opening width of the stigmata remains generally quite small" (p. 70). He adds that ". . . the stigmata in pure O_2 are more closed than in ordinary air," which suggests that here, as in the silkworm, fluttering is controlled by P_{O_2}. Fluttering also occurs in locusts (Hoyle, 1959). How widespread fluttering is in other orders and whether it is usually controlled by P_{O_2} remains to be seen.

11. *Prospects*

The main function of spiracular closing mechanisms is water conservation. From an evolutionary viewpoint, we may imagine that natural selection has favored the development of mechanisms that keep spiracles open just enough to permit the exchange of respiratory gases but otherwise restrict their aperture. Such a water-conserving mechanism might be expected to be best developed in insects with severe water problems, like diapausing pupae which live for long periods without imbibing (*cf.* Imms, 1957, p. 145). As Buck points out (1957, p. 77) discontinuous respiration is an example of such a well-developed spiracular mechanism. And, bursts of carbon dioxide are easily understandable in terms of the spiracular movements, which have been analyzed in earlier sections of this discussion. But what of the disparate rates of gas exchange between bursts, which are the real enigma of discontinuous respiration? Somehow during the interburst the spiracles manage to let oxygen enter, yet simultaneously prevent carbon dioxide and water vapor from leaving. Whilst the present experiments have not penetrated this secret, they nonetheless provide an experimental framework upon which a theory of mechanism must be based. For the heart of the problem surely lies in the flutter period, where hour after hour the insect practices the "trick" of filtering in oxygen, while retaining carbon dioxide and water. It is our belief that the real significance of the flutter period may have been overlooked in previous explanations of this peculiar one-way transfer of gases. The importance of the flutters in the kinetics of gas exchange will be discussed in a succeeding communication.

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SUMMARY

1. Experiments were conducted to examine the role of the spiracles in discontinuous respiration, to define the kinds of behavior that spiracles can show, and to clarify the manner in which tracheal P_{O_2} and P_{CO_2} interact to provoke various modes of spiracular activity. To accomplish this, records were made of the movements of the spiracular valves of diapausing pupae and developing adults of the Cecropia, Polyphemus and Cynthia silkworms, in air and in gas mixtures.

2. Spiracular valve movements in these silkworms occur in repeated cycles, with periods of from a few minutes to many hours. Each cycle consists of an open period or spiracular burst (which corresponds to the CO_2 burst), a closed or constriction period, and a flutter period which ordinarily occupies most of the cycle. In pupae with long cycles, the respiratory events occur virtually in slow motion when compared with other insects, and this permits careful analysis of complex events in gas exchange and spiracular behavior which are not readily separable in other insects. Evidence is presented that each spiracular act (fluttering, burst, valve closure) is a response to a specific chemical stimulus: the gaseous composition of the tracheal system.

3. In pure O_2, the flutter period is ordinarily eliminated. As ambient P_{O_2} decreases, fluttering reappears and the flutter period progressively lengthens, until, in P_{O_2}'s below 15%, the spiracular bursts disappear and fluttering is continuous.

4. Ambient P_{CO_2} ordinarily has no effect until it increases above 5%, whereupon the cycles shorten. This shortening occurs at the expense of the flutter period, which progressively diminishes as P_{CO_2} increases. When P_{CO_2} rises above about 15%, the cycles break down completely and the valves flutter continuously.

5. Intubating even one pupal spiracle eliminates the cycles in the remaining 13, and the valves stay permanently constricted, presumably because normal triggering stimuli for spiracular activity are absent.

6. The spiracles of intubated pupae can be caused to open by lowering the P_{O_2} or raising the P_{CO_2}. The P_{CO_2} which opens the spiracles varies with the ambient P_{O_2}: in a typical pupa, in 2.3% O_2, 5% CO_2 opened the spiracles, whereas in 4.7% O_2, 10% CO_2 was required.

7. From these and other data, it is concluded that the cyclical movements of the spiracles result from cyclical changes in tracheal composition. In particular, fluttering is initiated by low P_{O_2}, while spiracular bursts are caused by high P_{CO_2}.

8. Evidence is presented to prove that the pupa possesses an independent O_2-sensitive mechanism, which is quite separate from any CO_2-sensitive mechanism. It is also argued that low P_{O_2} does not affect spiracular behavior by virtue of anoxia-produced acidity, nor by virtue of acidity releasing bound carbon dioxide.

9. The spiracular behavior of these silkworms is compared in detail with the picture of spiracular behavior in the flea provided by Wigglesworth, and it is concluded that there is no fundamental difference between the two, except for the flutter period which seems peculiar to the pupa. Evidence is presented that the flutter period holds the key to the disparate rates of gas exchange between bursts, which remains the central problem of discontinuous respiration.

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FURTHER STUDIES ON THE TREMATODE GENUS HIMASTHLA WITH DESCRIPTIONS OF H. MCINTOSHI N. SP., H. PISCICOLA N. SP., AND STAGES IN THE LIFE-HISTORY OF H. COMPACTA N. SP.

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The genus *Himasthla* is a member of the Echinostomatidae, a family which comprises a large number of genera and species that live in the digestive tracts of birds and mammals. Dietz (1909) erected the genus with *H. rhigedana*, a new species from the curlews, *Numenius arquatus* and *Numenius arabicus*, as type. He (1910) characterized the family and the several genera. As members of *Himasthla* he included *H. militaris* (Rudolphi, 1803) from the European curlew, *Scolopax* (= *Numenius*) *arquata; H. leptosoma* (Creplin, 1829) from *Tringa alpina* (= *variabilis*); *H. elongata* (Mehlis, 1831) from species of gulls, *Larus; H. secunda* (Nicoll, 1906) from the black-headed gull, *Larus ridibundus* and the herring gull, *Larus argentatus;* and *H. alincia* Dietz, 1909, based on specimens from the semipalmated sandpiper, *Tringa pusillus* collected by Natterer in Brazil. The first of these species was taken on the Sinai peninsula, the last in South America, while the others were European. As important criteria for specific determination, Dietz listed the number of spines on the collar, the extent of the vitellaria, and the structural details of the copulatory organs.

Subsequently described species include *H. harrisoni* Johnston, 1917 from *Numenius cyanopus* in Australia; *H. incisa* Linton, 1928 from the white-winged scoter, *Oidemia deglandi*, at Woods Hole, Massachusetts; *H. muehlensi* Vogel, 1933 from a human patient in Hamburg, Germany; *H. ambigua* Palombi, 1934, based on encysted metacercariae from the gills of *Tapes decussatus* in the Mediterranean; *H. kusasigi* Yamaguti, 1939 from *Tringa ochlopus* and *H. megacotyle* Yamaguti, 1939 from *Erolia alpina sakhalina*, both species taken in Japan; *H. multilecithosa* Mendheim, 1940 from a captive great crowned pigeon, *Goura coronata;* and *H. tensa* Linton, 1940 reported from a codfish, *Gadus morrhua*, at Woods Hole, Massachusetts. Stunkard (1934, 1937, 1938) showed that *Cercaria quissetensis* Miller and Northup, 1926 is the larval stage of a species of *Himasthla*, for which the larval name was adopted. The asexual generations occur in *Nassarius obsoletus*, the cercariae penetrate and encyst in different species of mollusks, and the worms become sexually mature in the intestine of gulls. Prudhoe (1944) described, but did not name, a single specimen from the yellow-wattled lapwing, *Lobipluvia malabarica*, and assigned it provisionally to the genus *Himasthla*.

Metacercariae from the foot of *Scrobicularia tenuis* were identified as *Cercaria leptosoma* by Villot (1878), who traced their development to maturity in the alimentary tract of the "allouette de mer à collier," *Tringa variabilis*. The number of collar-spines was not recorded, but the figure of the adult is very similar to that of *H. leptosoma* as given by Dietz. Cuénot (1892) reported metacercariae of *H. leptosoma* encysted in the circumoral tentacles of *Synapta inhaerens* and in the foot of *S. tenuis*, but since he found 31 or 32 collar-spines, it is obvious that he was dealing with some species other than *H. leptosoma*. Palombi (1925) identified metacercariae from *Mytilus galloprovincialis* as larvae of *Echinostomum secundum* (= *H. secunda*) Nicoll, 1906. Skrjabin (1956) included figures of the larval stages of *H. militaris* after the work of Zelikman, but there was no account of the life-cycle or reference to other published report.

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There has been much difference of opinion concerning specific distinctions and the number of valid species in *Himasthla*. Stunkard (1939) reviewed the history of the genus and discussed the problem of specific determination. Linton (1928) had described specimens from four species of gulls (*Larus*) and others from the white-winged scoter, *Oidemia deglandi*, and from the black-crowned night heron, *Nycticorax nycticorax*. The worms from *O. deglandi* were described as a new species, *H. incisa,* while all the others were referred to *H. elongata*, despite differences in number of collar-spines and other morphological features. Some of the worms had 29, others 31 collar-spines, and it was clear that two distinct species were represented. Those with 31 spines were identified by Stunkard (1938) as *H. quissetensis*, whereas those with 29 spines were intermediate in size and morphology or overlapped the figures given by Dietz (1910) as characteristic for *H. elongata* and *H. militaris*. Stunkard (1938) stated, p. 190, "In my opinion the worms might with equal justification be referred to either of the two species. On the other hand, they may belong to neither." Sprehn (1932) listed *H. militaris* and *H. secunda* as synonyms of *H. leptosoma* and Palombi (1934) admitted the probable identity of *H. leptosoma* and *H. secunda*. Stunkard noted similarity between *H. alincia* and *H. quissetensis* and conceded their possible identity. Dawes (1946) regarded *H. leptosoma* and *H. elongata* as valid species, but listed *H. militaris* and *H. secunda* as synonyms of *H. leptosoma*.

In correspondence with Dr. Vogel, Dr. Mendheim accepted the opinion of Sprehn (1932) and in addition predicated the identity of *H. elongata* and *H. leptosoma*. With this proposal, all the specimens of *Himasthla* with 29 collar-spines were included in a single species, *H. elongata*. However, if the idea were correct, the name of the species must be *H. militaris* (Rudolphi, 1803), which has priority over *H. elongata* (Mehlis, 1831). In addition, Dr. Mendheim suggested the identity of *H. muehlensi* and *H. elongata*. The specimens of *H. muehlensi* had been recovered from the stool of a patient following a purgative after his arrival at the hospital of the Institut für Schiffs- und Tropenkrankheiten in Hamburg, Germany. The patient was a South American who had stopped at New York on his way to Germany and in sea-food restaurants had eaten many "clams," juvenile *Venus* (*Mercenaria*) *mercenaria* which are served raw on the half-shell under the designation "cherry stones." In his description of the specimens, Vogel (1933) noted that members of *Himasthla* are typically parasites of birds and that the human infection was a recent and probably an accidental one. In a monographic study of the Echinostomidae, Mendheim (1940) described *Himasthla multilecithosa* n. sp., from *Goura coronata*, a native of the Papuan and Solomon Islands. Mendheim reported that the time and place of the infection were unknown, that the normal food of these birds is fruit, and since the infective larvae of *Himasthla* occur in marine mollusks, the infection must have been accidental or incidental. After redescriptions of *H. elongata* and *H. muehlensi* he wrote, p. 578, "Auf Grund einiger Befunde müssen *Himasthla leptosoma, Himasthla elongata* und *Himasthla muehlensi* zu einer Art vereinigt werden." But his account of *H. elongata* was obviously based on material of more than one species since he reported the number of collar-spines as 29–31. His report on the specimens of *H. muehlensi*, loaned by Dr. Vogel, adds nothing of significance to the original description. He noted that egg production had just begun and that the first eggs were smaller than those formed later, from which he concluded, p. 511, "Die Eimasse können also nicht als brauchbares Artkriterium gelten." The observation is not new (q.v., Beaver, 1937, p. 26) and the conclusion is equivocal.

The writer had the opportunity in Hamburg to study the specimens of *H. muehlensi*, several specimens of *H. leptosoma* from *Tringa alpina* in the collection of Dr. Vogel, and four specimens sent by Dr. Mendheim that were identified as *H. elongata*. In a report, Stunkard (1939) stated that the specimens of *H. muehlensi* have 31 collar-spines and while there is a possibility that they may be identical with *H. quissetensis*, they certainly are distinct from *H. elongata* which has 29 spines. Furthermore, although *H. elongata* and *H. leptosoma* both have 29 collar-spines, morphological differences, first listed by Dietz and manifested by specimens from the collections of Vogel and Mendheim, clearly differentiate the two species. Specimens of *H. leptosoma* are smaller, the structure is more delicate, the suckers, collar-spines and eggs are smaller, the vitellaria do not extend as far anteriad, and the gonads are larger. Mendheim (1943) reaffirmed the identity of *H. elongata, H. leptosoma* and *H. muehlensi*, which he derived from average measurements of 15 specimens which, according to the text, included the four worms identified by Stunkard as *H. elongata*. Since the author admittedly was unable to distinguish between *H. elongata* and *H. leptosoma*, and since it is apparent from his figures that the 15 specimens included representatives of both species, the average measurements are meaningless. In a key for determination of species, Mendheim (1943, p. 235) distinguished *H. incisa* on the basis of 27 collar-spines. In the original description Linton (1928) wrote, p. 12, "The exact number of oral spines could not be made out in the balsam mount. There are at least 27. There is a single row, except at the lateral angles." After examination of the type specimen, Stunkard (1938) reported that the worm has 31 spines. Re-examination of the specimen confirms that number; the spines are relatively stout and those in the single row measure 0.065 to 0.070 mm. in length. The slide bearing the single type specimen has a label written by Professor Linton noting that the worm was collected by Vinal Edwards, June 2, 1914, at Woods Hole, Massachusetts.

Linton (1940) described *H. tensa* as a parasite of *Gadus morrhua;* there are three specimens on the slide deposited in the U. S. National Museum and the notation, in Linton's handwriting, states that the worms were collected by Vinal Edwards, 1915. Mendheim apparently accepted the fish as a normal host. He stated, (1943, p. 235) "Besonders bemerkungswert ist *H. tensa* aus *Gadus morrhua*. Es ist dies seit dem von Diesing beschriebenen *Echinostoma annulatum* Diesing 1850 die einzige Echinostomiden-Art aus Fischen. Körperform, Lage des Bauch-

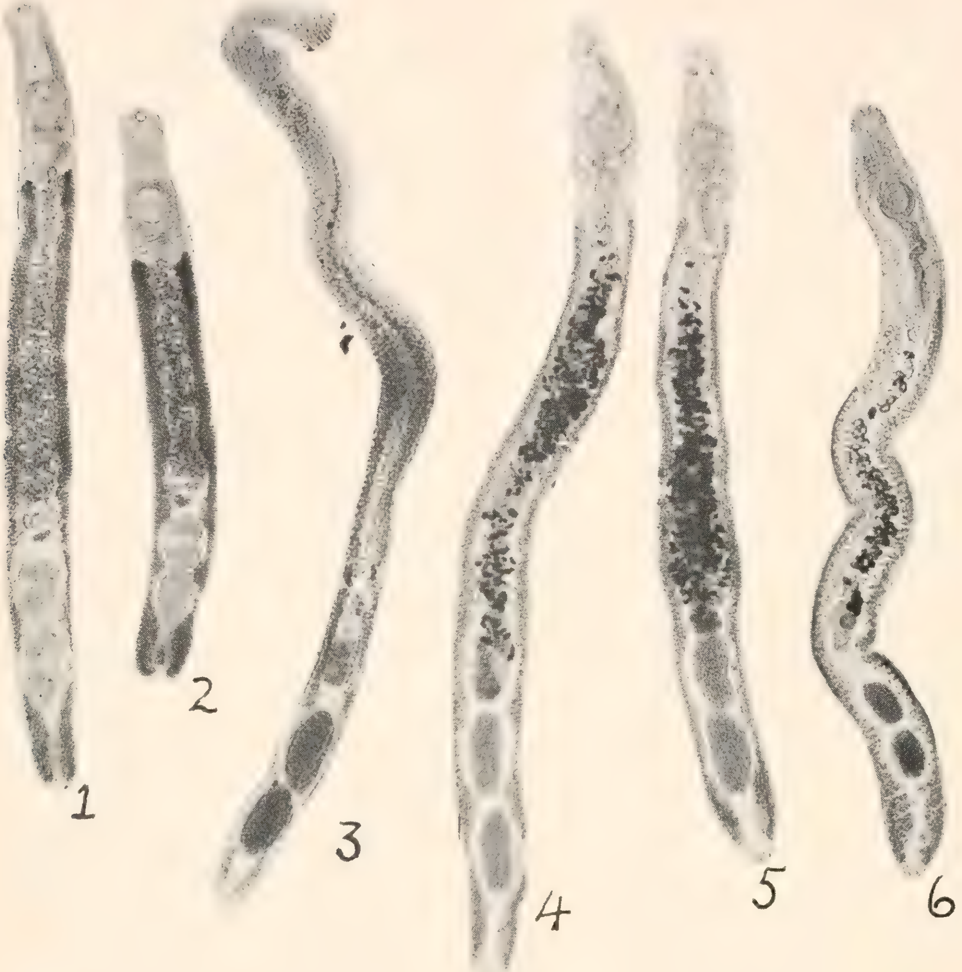

PLATE I

FIGURE 1. *Himasthla compacta,* type specimen; length 4.00 mm.

FIGURE 2. *Himasthla compacta,* cotype specimen; length 3.10 mm.

FIGURE 3. *Himasthla elongata,* juvenile specimen without eggs, from *Larus delawarensis,* taken 24 January 1914 at Woods Hole, Mass., by Vinal Edwards. Linton material now in U. S. National Museum Helminthological Collection, No. 7922; length 6.00 mm.

FIGURE 4. *Himasthla elongata,* from *Larus argentatus,* taken 18 December 1921 at Woods Hole, Mass., by R. A. Goffin. Linton material now in U. S. National Museum Helminthological Collection, No. 7921; specimen 8.75 mm. long.

FIGURE 5. *Himasthla elongata,* on same slide as Figure 4, same data; specimen 6.75 mm. long.

FIGURE 6. *Himasthla elongata prov.,* from night heron, *Nycticorax nycticorax,* taken 11 September 1914 at Woods Hole, Mass., by Vinal Edwards. Linton material now in U. S. National Museum, No. 7924; specimen 6.00 mm. long.

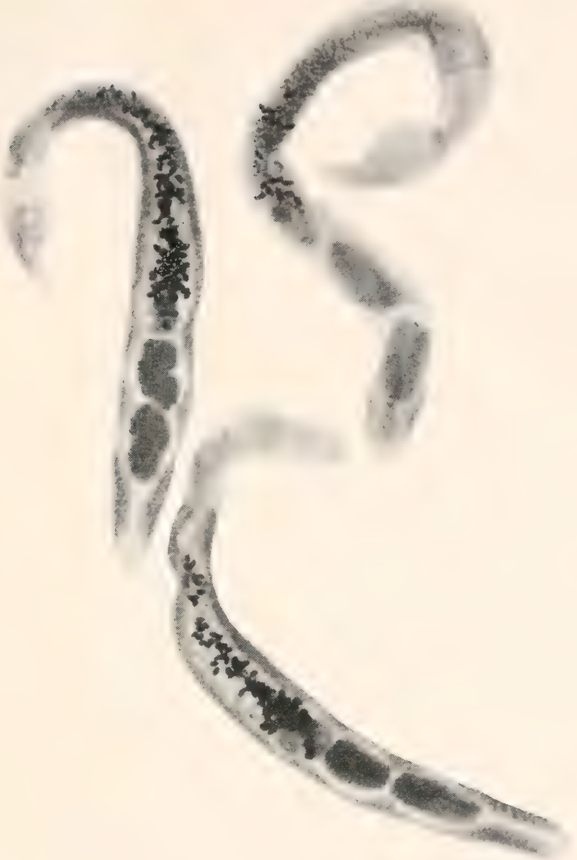

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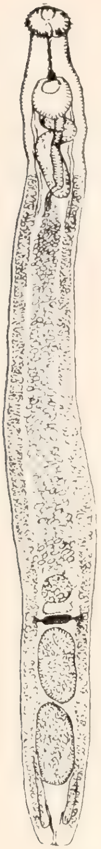
PLATE II

Figure 7. *Himasthla tensa* Linton, 1940. Type and cotype specimens from *Gadus morrhua,* taken 22 January 1915 at Woods Hole, Mass., by Vinal Edwards. Linton material now in the U. S. National Museum, No. 8214; type specimen at bottom of photograph; length, 5.32 mm.

saugnapfes und Pseudosegmentierung lassen es vor allem seit Linton's Fund als durchaus wahrscheinlich erscheinen, dass Diesing eine *Himasthla*-Art vorgelegen hat." *Distomum annulatum* was described by Diesing (1850) from the intestine of the electric eel, *Gymnotus electricus.* The specimens had been collected by Natterer in Brazil, the 3rd of September, 1827. In his description and figures, Diesing (1855) gave the length as 6 lines and the maximum width as ½ line, which would be slightly more than 12 by 1 mm. Cobbold (1860) transferred the species to *Echinostoma* and Dietz (1910) listed it under *Species inquirendae.* Mendheim noted features in which it conforms to the diagnosis of *Himasthla.* The specific name, *annulatum,* is significant since this feature is a prominent characteristic of the genus. Examination of the figures of Diesing (1855) and comparison



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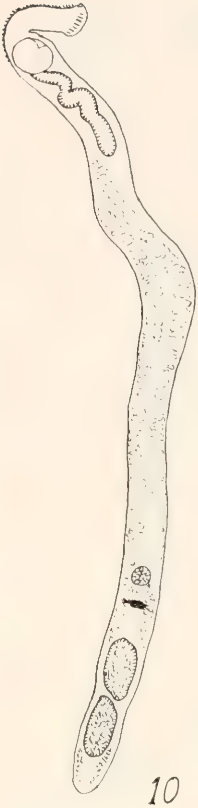

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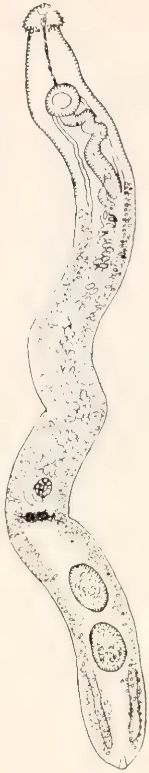

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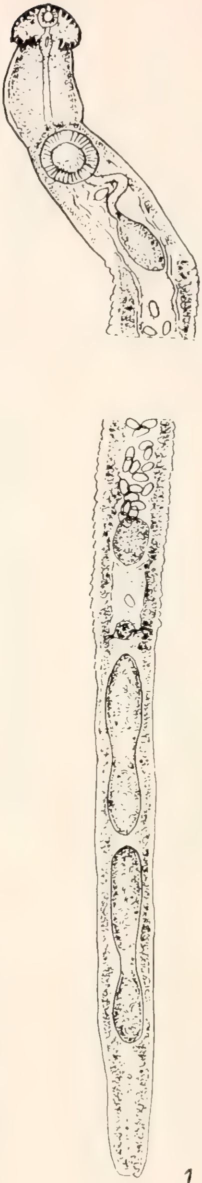

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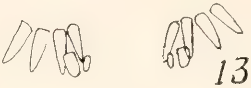

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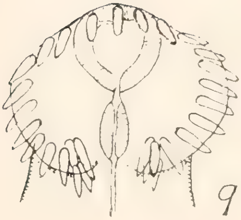

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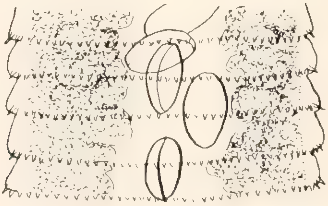

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PLATE III

FIGURE 8. *Himasthla elongata,* drawing of specimen shown in Figure 5.
FIGURE 9. Collar and spines of specimen shown in Figure 8.
FIGURE 10. *Himasthla elongata,* drawing of specimen shown in Figure 3.

of his Figure 21, Plate III, showing five annuli, with Figure 14 of the present work, show that the worms described as *D. annulatum* agree far better with the generic concept of *Himasthla* than with that of *Echinostoma*. Accordingly they are transferred to that genus as *Himasthla annulata* (Diesing, 1850). Although *H. annulata* and *H. tensa* were described from fishes, each species has been found only once and there are strong reasons for regarding both of them as parasites of birds which were found incidentally in the intestine of the electric eel in Brazil and the codfish of New England, respectively.

MATERIAL AND METHODS

This report presents results from one section of the investigation conducted by the U. S. Bureau of Commercial Fisheries to discover causes for the decline in number of soft-shelled clams, *Mya arenaria*, along the coast of New England. To Walter R. Welch, Chief, Clam Investigations, and his staff in Boothbay Harbor, Maine, grateful appreciation is expressed for avian and molluscan specimens provided for the study. Other material was provided by the Marine Biological Laboratory, Woods Hole, Massachusetts, where the experimental work was done. Examination of clams from the region of Boothbay Harbor disclosed echinostome metacercariae encysted in the palps and gills. These larvae were fed over periods of one to seven weeks to laboratory-reared eider ducks (*Somateria mollissima*), herring gulls (*Larus argentatus*), terns (*Sterna hirundo*), white mice and golden hamsters. The worms excysted in eider ducks but did not persist more than about ten days. Adult worms were recovered from only one species, *L. argentatus*. Further study has shown that three species, all members of the genus *Himasthla*, are concerned. One of these species, *H. quissetensis*, was described by Stunkard who (1938) found the asexual generations in the mud-snail, *Nassarius obsoletus;* the life-history of another, described as *H. compacta* n. sp., is reported in the present paper; while the third species is identified as *H. elongata* (Mehlis, 1831). All three species have been recovered from the intestine of laboratory-reared gulls, *L. argentatus*, fed palps and gills of *M. arenaria* collected in the Boothbay Harbor area. To preclude accidental and complicating infections, the birds were fed commercial, canned cat-food, made from fishes and supplemented with vitamins. The examination of shore-birds in the Boothbay Harbor area disclosed a fourth species, identified as *H. alincia*, in the intestine of *Ereunetes* (*Tringa*) *pusilla*. This is the host from which the species was originally described by Dietz (1909).

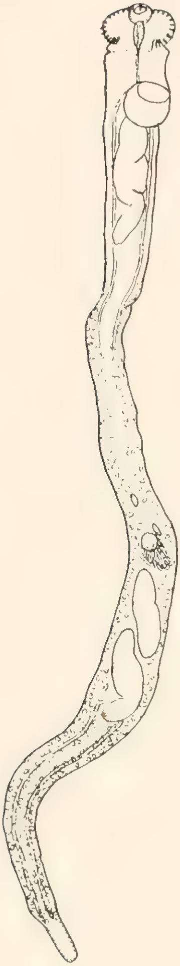
To supplement the study of these worms, all specimens of *Himasthla* in the Helminthological Collection of the U. S. National Museum have been received on loan through the kindness of Dr. Allen McIntosh and the favor is here gratefully acknowledged. The collection contains several specimens from *Larus argentatus*

FIGURE 11. *Himasthla elongata*, drawing of specimen shown in Figure 6.

FIGURE 12. *Himasthla elongata*, a greatly extended specimen from the intestine of *Larus argentatus*, experimental infection; the bird had been fed metacercariae encysted in the gills and palps of *M. arenaria*. In the drawing, a section 6 mm. long, containing 110 annuli, is omitted from the middle of the body; specimen 12.5 mm. long.

FIGURE 13. Spines at the ventro-lateral corners of the collar; note the very small median spines.

FIGURE 14. Five annuli from the central portion of the specimen shown in Figure 12.



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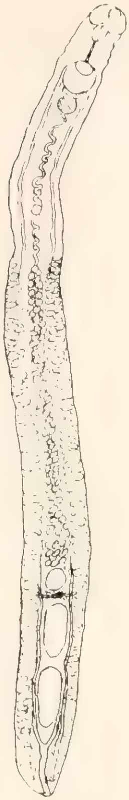

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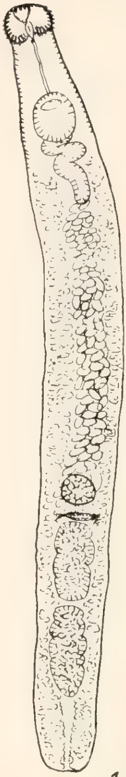

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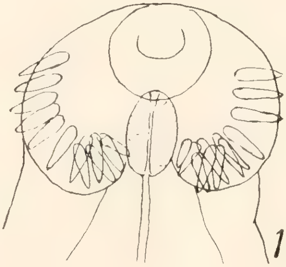

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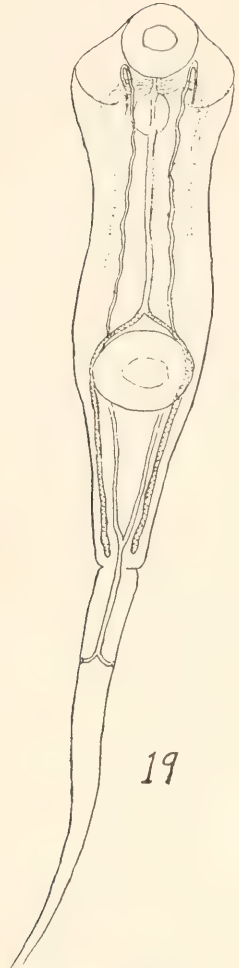
PLATE IV

Figure 15. *Himasthla piscicola,* type specimen, length 9.80 mm.
Figure 16. *Himasthla mcintoshi,* type specimen, length 8.00 mm.
Figure 17. Spines at the ventro-lateral corners of the collar of worm shown in Figure 16.
Figure 18. *Himasthla compacta,* drawing of specimen shown in Figure 1.

and one from *Nycticorax nycticorax* which had been identified by Linton as *H. elongata.* It contained, also, the type material of *H. incisa* Linton, 1928 and *H. tensa* Linton, 1940. Examination of the specimens of *H. tensa* discloses that the number of collar-spines is 29, not "about 32" as reported by Linton (1940). In the three specimens (Fig. 7), the vitelline follicles terminate a short distance posterior to the caudal end of the cirrus-sac. In the specimens of *H. elongata* shown in Figures 4 and 5, the vitellaria extend anteriad slightly past the caudal end of the cirrus-sac but in other individuals, especially younger ones, the vitellaria do not extend to the level of the cirrus-sac. No constant differences were found between these worms and, accordingly, the name *H. tensa* is suppressed as a synonym of *H. elongata.* The Museum Collection also contains four slides bearing the number 54,721, with seven specimens from the "long-billed curlew," *Numenius americanus americanus,* collected by J. Bushman at Orr's Ranch, Tooele Co., Utah, April 21, 1954. These worms have 35 collar-spines and are described as a new species, *Himasthla mcintoshi.* Subsequently, Dr. George R. La Rue of the Research Center, U. S. Department of Agriculture, Beltsville, Maryland, sent two specimens taken from the digestive tract of a South American fish, *Arapaima gigas,* that died in the Toledo, Ohio, zoo and that had been referred to him for identification. Grateful acknowledgment is made also to Dr. La Rue for the privilege of examining and describing these worms.

To locate the asexual generations of the metacercariae which occur in the gills and palps of *M. arenaria,* a survey of the mollusks in the Boothbay Harbor area was begun. As noted above, *N. obsoletus* had been identified as the first intermediate host of *H. quissetensis.* The first intermediate hosts of *H. elongata* and *H. alincia* are yet unknown, but there is strong and almost conclusive evidence that an echinostome cercaria from *Hydrobia minuta* is the larval stage of one of the metacercariae in *M. arenaria* and that this metacercaria develops in *L. argentatus* to adults described in this paper as *Himasthla compacta* n. sp. *Hydrobia minuta* harbors at least six different species of cercariae. Examination of 5000 snails, isolated 100 per dish, yielded three specimens shedding an echinostome cercaria and examination of 5000 snails in another series, in which an individual count was kept as the snails were crushed and examined under the microscope, disclosed six infections by this echinostome cercaria. It has 29 collar-spines and is the only echinostome cercaria, other than that of *H. quissetensis* which has 31 spines, found so far in the region. Very small specimens of *M. arenaria* collected in the Woods Hole, Massachusetts, area were added to the dishes containing the cercariae from *H. minuta* and metacercariae, presumably of experimental infection, were recovered from them. Owing to the low incidence of infection and the small size of the snails, and the resulting scarcity of cercariae, it has not been possible to produce massive infections which are easily obtained with the abundant cercariae of *H. quissetensis.* When using clams collected in the field, there is the possibility that they may be carrying metacercariae of natural infection, but examination of 200 specimens from the area where those used in the experiment were taken, revealed no infection. Since laboratory-reared clams were not available, very small clams from the Woods Hole area were employed. These clams, exposed to the cercariae, were fed subsequently to a young, laboratory-reared gull, *L. argentatus.* The bird began to pass trematode eggs some four weeks after the first feeding and later, on examination, it contained 26 small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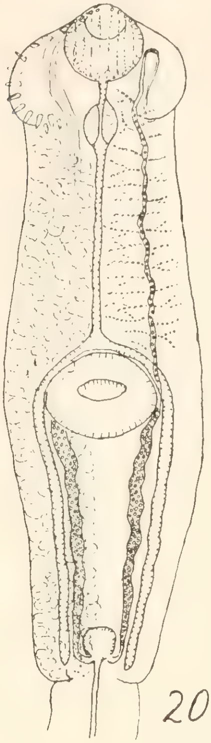
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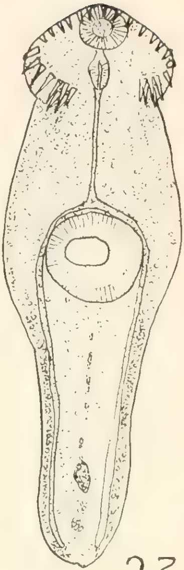
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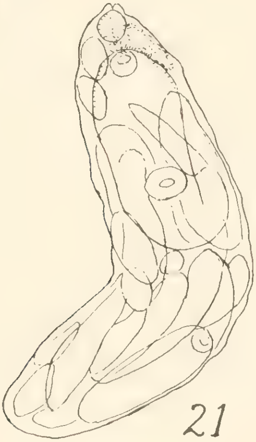
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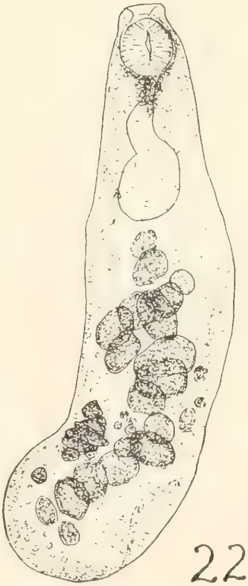
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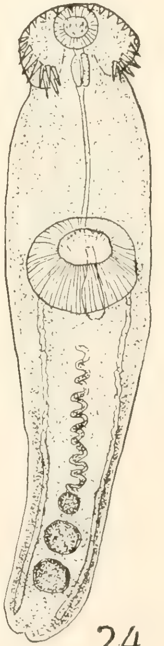
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PLATE V.

echinostomes, identified as *H. compacta* n. sp. This species has 29 collar-spines and identification of the larval stages is not easy. The sizes of the cercariae and of their metacercariae are not significantly different from those of *H. quissetensis* and do not provide certain criteria for specific determination of the larvae. The adults are distinguished easily. Consequently, it appears that one of the best means of positive identification is to expose small clams to the cercariae and then feed the metacercariae to previously unexposed birds.

Additional data on the life-cycle are provided by results of attempts to infect the snail host. Eggs of *H. compacta*, taken from worms that had developed in a laboratory-reared gull, were incubated in sea-water at room temperature. At the end of four weeks they contained fully formed miracidia and were added to a finger-bowl containing 20 juvenile *H. minuta*. Four weeks later the number of living snails was reduced to 13. Snails that died were not examined, since the tissues decomposed quickly and trematode larvae would probably not be recognizable. One snail, dissected four weeks after exposure, contained three small rediae (Fig. 22) but whether they were mother or daughter rediae was not determined. No cercariae were obtained and the experiment was disappointing; however, it illustrates the difficulties inherent in this type of research. The number of eggs was limited, development seemed to proceed at variable and inconstant rates, and actual hatching was not observed. Since individual exposure of a snail to one or more miracidia could not be made, the mass exposure method was undertaken to determine whether or not certain of the miracidia on emergence could find and infect snails. With such "shotgun" technique, it is impossible to know how many, if any, miracidia penetrated a particular snail and it is possible that the death of certain snails was caused by superinfection. The one experimental infection, however, supplements other data and strongly supports the postulated life-cycle.

DESCRIPTIONS

Himasthla elongata (Mehlis, 1831)

Linton (1928) described trematodes from *Larus argentatus* at Woods Hole, Massachusetts, which he identified as *Himasthla elongata*. Stunkard (1938) pointed out that the material of Linton comprised two distinct species, one of which was identified as *Himasthla quissetensis,* whose cercarial stage had been described

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PLATE V

FIGURE 19. *Himasthla compacta,* young cercaria from *Hydrobia minuta,* natural infection, specimen fixed and stained; body 0.50 mm. long; tail 0.37 mm. long; to show digestive, nervous and excretory structures.

FIGURE 20. *Himasthla compacta,* normally emerged cercaria, sketch of living specimen to show glands and excretory ducts.

FIGURE 21. *Himasthla compacta,* redia, natural infection, fixed and stained; specimen 1.12 mm. long.

FIGURE 22. *Himasthla compacta,* redia, experimental infection, fixed and stained; specimen 0.47 mm. long.

FIGURE 23. *Himasthla compacta,* young specimen from gull, fixed and stained; 0.46 mm. long.

FIGURE 24. *Himasthla compacta,* young specimen from gull, fixed and stained; 0.76 mm. long.

and named by Miller and Northup (1926). The members of the other species were not identified although Stunkard noted that measurements of 12 representative specimens are intermediate between or overlap the figures given by Dietz (1910) as characteristic for *H. elongata* and *H. militaris*.

Certain of the adult worms recovered from the intestine of *L. argentatus* after feeding metacercariae encysted in the tissues of *M. arenaria* from Maine are identified as *H. elongata*. These worms unquestionably are specifically identical with those identified as *H. elongata* by Linton (1928). In most of the specimens the collar-spines are slightly larger and the testes considerably larger than the figures given by Dietz (1910) for *H. elongata* but there is considerable variation, and the location of the testes and ovary near the posterior end of the body clearly differentiates them from *H. militaris*. One specimen, killed in a very extended condition (Figs. 12, 13, 14), exhibits the pseudosegmentation characteristic of the postacetabular region of the body and the annular arrangement of the flattened, scale-like spines. In this specimen, the two median members of the corner-spines (Fig. 13) on the collar are very small, recalling the condition shown in Figure B^1 of Dietz. The worm measures 12.5 mm. in length and 0.45 mm. in greatest width. In Figure 12, 6 mm. and 110 annulations are omitted from the middle of the body. Five of the annuli are portrayed in Figure 14. Another, younger specimen, with a few eggs in the uterus gave the following measurements: length, 4.4 mm.; width, 0.5 mm.; width at collar, 0.3 mm.; spines, 0.058 mm. long; acetabulum, 0.31 mm. long and 0.26 mm. wide; oral sucker, diameter 0.11 mm.; pharynx, 0.098 mm. long and 0.056 mm. wide; vitellaria do not extend forward to the level of the cirrus-sac; ovary 0.156 mm. wide and 0.125 mm. long; anterior testis, 0.25 mm. long and 0.155 mm. wide; posterior testis, 0.28 mm. long and 0.15 mm. wide; eggs 0.090 to 0.100 mm. long and 0.060 to 0.068 mm. wide. Gravid worms vary in size from these measurements to older individuals that are twice as large with correspondingly larger organs, but the structural pattern of the species is always evident.

The single specimen from the night heron, *N. nycticorax*, shown in Figures 6 and 11, has 29 collar-spines but manifests certain differences from the worms found in *L. argentatus*. It is only slightly smaller than the worm shown in Figure 5; however, the collar-spines and suckers are smaller, the vitellaria do not extend as far anteriad, and the gonads are smaller and slightly farther forward. In this specimen the spines are 0.050 mm. long; the oral sucker measures 0.114 by 0.107 mm.; the acetabulum is 0.325 mm. long and 0.290 mm. wide; the ovary is spherical, 0.143 mm. in diameter; the anterior testis is 0.40 mm. long and 0.23 mm. wide; the posterior testis is 0.42 mm. long and 0.25 mm. wide; the eggs average 0.097 by 0.062 mm. The measurements are almost identical with those given by Nicoll for *Himasthla secunda*, and the small size of the gonads agrees well with the description of *H. elongata* as given by Dietz. The similarity suggests possible identity of *H. secunda* and *H. elongata*. Since the material at hand consists of a single specimen, it is referred for the present to *H. elongata*. When the life-history of that species is known and cercariae are available for experimental infections, it will be possible to determine definitely whether the present worm belongs in *H. elongata* or is a member of some other species.

The specimen from *Larus delawarensis* (Fig. 3) is referred to *H. elongata*, but it is juvenile and possibly members of that species do not attain sexual maturity in *L. delawarensis*.

Himasthla compacta n. sp.

Adult

Material of this species consists of worms recovered from laboratory-reared *L. argentatus* fed *M. arenaria* collected near Boothbay Harbor, Maine, and others from another laboratory-reared *L. argentatus*, five weeks after the beginning of an experiment in which the bird was fed metacercariae encysted in small *M. arenaria*. These clams had been exposed to echinostome cercariae from *H. minuta* collected in Sagadahoc Bay, near Boothbay Harbor, Maine, and the metacercariae were presumed to be encysted stages of the same cercariae. Most of the worms were sexually mature but several were juveniles. The bird had been fed five weeks, twc weeks and one week before it was killed and the small worms shown in Figures 23 and 24 are probably from the last two feedings. A specimen 2.130 mm. long and 0.275 mm. wide does not have eggs in the uterus, although there are spermatozoa in the testes.

Gravid specimens, fixed and stained, measure 3.00 to 4.30 mm. in length and 0.35 to 0.44 mm. in width. For such a small species of *Himasthla*, the organs are large and compactly disposed. The sides of the body are almost parallel; the acetabulum protrudes; in the preacetabular region the lateral edges are often turned ventrad and mediad, forming a ventral depression. The anterior end has a reniform collar, open ventrally, which bears 29 spines, 25 arranged in a linear row and two smaller corner-spines on each side behind the terminal ones of the row. The lineal spines are 0.054 to 0.062 mm. in length and 0.012 to 0.014 mm. wide; the smaller corner ones are 0.026 to 0.032 mm. long and 0.009 mm. wide. In mature specimens the acetabulum is about one-seventh of the body length from the anterior end whereas in juvenile worms it is relatively farther back and in young worms it is near the middle of the body. The shift in relative position of the acetabulum results from development of the reproductive organs in the postacetabular portion of the body. The acetabulum is usually longer than broad, oriented with the opening at the antero-ventral face, just behind the common genital pore. The sucker measures 0.20 to 0.26 mm. in length and 0.18 to 0.22 mm. in width. The cuticula in the preacetabular area bears scale-like spines arranged in an imbricate pattern, while behind the sucker the spines are smaller and are arranged in the annular fashion characteristic of the genus.

The mouth is subterminal; the oral sucker measures 0.075 to 0.090 mm. in diameter. There is a short prepharynx; the pharynx is oval to pyriform, usually wider posteriorly, it measures 0.060 to 0.075 mm. in length and 0.040 to 0.050 mm. in width. The esophagus extends almost to the acetabulum and the ceca end blindly near the posterior end of the body.

The testes are almost contiguous, one behind the other, in the caudal one-third of the body. They are oval, with notched but not lobed surfaces. The posterior testis is usually somewhat larger than the anterior one. The anterior testis is 0.36 to 0.49 mm. in length and 0.18 to 0.21 mm. in width; the posterior testis is 0.40 to 0.58 mm. in length and 0.18 to 0.21 mm. in width. From each testis a sperm duct passes forward and the two unite just before reaching the cirrus-sac. The common duct, on entering the sac, expands into a coiled seminal vesicle, which fills the posterior one-half or more of the cirrus-sac. The vesicle is followed by a shorter prostatic portion of the duct and then by a protrusible cirrus armed with very small spines. The cirrus-sac extends behind the acetabulum more than the diameter of that sucker and terminates between the anterior ends of the vitelline glands.

The ovary is spherical to oval, usually broader than long, situated from two-sevenths to three-eighths of the body length from the posterior end. It is about its diameter in front of the anterior testis. It measures 0.06 to 0.13 mm. in length and 0.08 to 0.16 mm. in width. The oviduct arises at the posterior face and passes backward where it enters the ootype region. It expands somewhat and gives off Laurer's canal, which passes to the dorsal surface of the body, after which it receives the common vitelline duct. This portion is partially enclosed in the cells of Mehlis' gland, which surrounds the ootype. The initial portion of the uterus is filled with masses of spermatozoa. The uterus coils about and passes forward to the level of the caudal end of the cirrus-sac where it joins the metraterm. Both metraterm and cirrus-sac pass forward above the acetabulum to open into a shallow genital sinus, and the genital pore is on the median ventral surface, immediately anterior to the acetabulum. The vitelline follicles are lateral to and partially overlap the digestive ceca; they extend from the caudal end of the body to a level about the length of the acetabulum behind that sucker. They are not interrupted at the levels of the testes. Longitudinal ducts connect the follicles and transverse ducts pass mediad at the level of the ootype, where they unite to form a vitelline receptacle from which a common duct leads to the initial portion of the ootype. The eggs are large, oval, thin-shelled, collapsed in the preserved specimens, 0.085 to 0.090 mm. in length and 0.050 to 0.058 mm. in width. Each egg contains an ovum and several vitelline cells. The ovum is situated toward the opercular end of the egg. Cleavage begins in the uterus, but development there does not go much beyond the four-cell stage.

Himasthla compacta differs from all other species of the genus in its smaller size, its compact structure and relatively larger gonads. The ovary is situated about one-third of the body length from the posterior end and the testes extend through most of the postovarian distance, a condition not found in any other species. Since *H. compacta* has not been reported previously from *L. argentatus*, some other avian species may be its normal host.

The type specimen (Figs. 1, 18) is deposited in the U. S. National Museum Helminthological Collection under the number 39,444.

Redia

In structure and behavior, the rediae are very similar to those of *H. quissetensis* as described by Stunkard (1938). Figure 21 is a drawing of a large, gravid redia of natural infection. It is 1.12 mm. long and 0.28 mm. wide; the pharynx is 0.028 mm. in diameter. Figure 22 shows a young redia recovered from one of the small specimens of *H. minuta* which had been exposed for four weeks to embryonated eggs of *H. compacta* taken from worms that had developed in the intestine of a laboratory-reared specimen of *L. argentatus*. Whether this is a first or second generation redia could not be determined, since the germ balls in it could be embryos of either rediae or cercariae. At this stage they are quite indistinguishable. The specimen measures, fixed, stained and mounted, 0.47 mm. long, 0.12 mm. wide, and the pharynx is 0.027 mm. in diameter. The young rediae have collars which become visible as the larvae move; they progress in a lumbricid manner, with temporary protrusions of foot-like projections to anchor one region while advancing another. In young rediae the gut is conspicuous, filled with yellowish-green material, obviously the residue from digestion of snail tissue. The intestine does not increase in size and becomes proportionately smaller as the cavity of the redia becomes filled with larvae of the next generation. With increase in size and accumulation of growing progeny, the rediae become less and less active. Meanwhile, with advancing maturity, the cercariae become more vigorous in their movements until they emerge through the birth-pore, situated near the pharynx.

Cercaria

The incidence of infection of *H. minuta* is low, about one in five hundred, and cercariae emerged from less than one-half of the infected snails. The snails are small and few cercariae are produced. They leave the rediae and complete development in the haemocoele of the snail. In a crushed snail with mature infection, usually there are two to four or five cercariae free in the sinuses and others still in rediae. Some of those in the rediae swim actively when released, but the small number of cercariae restricts experimental procedure. In swimming, the body is contracted until it is almost spherical while the tail is extended and lashes vigorously.

The body in living cercariae measures 0.30 to 0.60 mm. in length and 0.08 to 0.19 mm. in width; it is elongate oval in outline although the shape varies with the degree of elongation and retraction. The collar gives the anterior end a characteristic form and when the larva is fully extended, this may be the widest part of the body. The tail is shorter than the body and capable of great extension and retraction by contraction of the circular or longitudinal muscles which form its wall. In naturally emerged cercariae the oral sucker measures 0.057 to 0.065 mm. in diameter; the ventral sucker 0.072 to 0.086 mm.; and the pharynx 0.014 to 0.020 mm. Figure 19 is of a well extended young cercaria from a crushed snail, fixed with hot AFAG (alcohol-formalin-acetic acid-glycerin) fixing fluid added to a small amount of sea-water in a beaker in which the larvae were being whirled. Stained and mounted, it has a total length of 0.87 mm.; the body is 0.50 mm. long and 0.13 mm. wide at the collar; the acetabulum is 0.080 mm., the oral sucker is 0.057 mm., and the pharynx is 0.020 mm. in diameter. The tail is 0.037 mm. wide at the base. The body of the cercaria contains three types of glandular cells. There are three pairs of penetration glands situated in the preacetabular area, and four pairs of glands in the oral sucker, all of which open through pores at the anterior end of the body. The entire dorsal area of the body is occupied by cystogenous cells whose cytoplasm is filled with bacilliform granules. These granules do not stain with vital dyes. Other glandular cells, more ventral in location, stain faintly with eosin and erythrosin.

The reproductive organs are represented by two groups of deeply staining cells, one near the posterior end of the body and the other at the anterior margin of the acetabulum, and a strand of cells extending between the larger groups. The posterior group is the rudiment of the gonads and the anterior one of the copulatory organs. Only the major portion of the excretory system was observed. In immature larvae the tissues are fragile and disintegrate before the tubules are visible and in older ones the excretory ducts are obscured by the masses of glandular cells. Certain observations indicate that the flame cells are arranged in groups of three, but the complete pattern was not worked out. The system forms as separate left and right components which meet and fuse at the posterior end of the body and the anterior portion of the tail. The two excretory pores are located on the sides of the tail, as shown in Figure 19. The vesicle is spherical to oval, thin-walled, and from either side a collecting duct passes forward, median to the digestive cecum, below the cecum at the level of the acetabulum, and then forward to the level of the oral sucker, where it recurves and passes posteriad. Near the middle of the body the recurrent duct divides into anterior and posterior branches. In the preacetabular area the collecting ducts have median and lateral branches and these branches subdivide in turn to form a ramified pattern as shown in Figure 20. In mature cercariae the collecting ducts contain spherical to oval concretions; in the postacetabular area the granules are larger, 0.005 to 0.01 mm. in diameter and more numerous, four or five at any level of the duct, whereas in the preacetabular ducts and their branches the granules are smaller, 0.003 to 0.005 mm. in diameter, and are arranged in single rows.

Himasthla mcintoshi n. sp.

This name is proposed for the seven specimens from the long-billed curlew, *Numenius americanus americanus*, collected by J. Bushman in Tooele County, Utah, on April 21, 1954 and deposited in the Helminthological Collection of the U. S. National Museum under the number 54,721. The species is named in honor of Dr. Allen McIntosh, Parasitologist in the Agricultural Research Service of the U. S. Department of Agriculture, in recognition of his contributions to helminthology and of the generous aid he has provided for other workers.

The worms are all sexually mature, with eggs in the uteri, chiefly in the initial one-half of the organ, with the terminal portion almost if not quite empty. Five of the worms are much bent or coiled and the one shown in Figure 16 is the only straight-bodied specimen. They vary from 6 to 11 mm. in length and from 0.5 to 0.7 mm. in greatest width. The anterior end bears a reniform collar which, in the two specimens in which they could be counted, is armed with 35 spines. There is a single row, interrupted ventrally, with 29 large spines, each 0.078 to 0.084 mm. in length and 0.02 mm. wide at the base, and at either end of the row, on the ventral side, there are three smaller corner-spines, about 0.055 mm. in length and 0.016 mm. wide at their bases. The lateral edges of the preacetabular region are curved ventrad and mediad, creating a median ventral depression (Fig. 17). When the body is contracted, the lateral walls are crenated and each annulus in the postacetabular region bears a circlet of small cuticular spines. In the preacetabular region the spines are closer together and arranged in an imbricated pattern, a cuticular arrangement characteristic of the genus *Himasthla*. The acetabulum is slightly less than twice its diameter from the anterior end of the body; it is directed antero-ventrad, protrudes slightly, and measures from 0.33 to 0.39 mm. in length and 0.31 to 0.35 mm. in width.

The mouth is subterminal, the oral sucker is 0.13 to 0.16 mm. in diameter, followed almost immediately by the pharynx, about 0.14 mm. long and 0.10 mm. wide. The esophagus extends almost to the acetabulum and the digestive ceca terminate blindly near the posterior end of the body.

The excretory pore is terminal, the vesicle is short and divides behind the posterior testis, with the collecting ducts passing forward just median to the digestive ceca. They are clearly visible in the region between the anterior ends of the vitellaria and the acetabulum. Anterior to the acetabulum they are lateral in position and extend forward to the level of the oral sucker where they turn backward. Further details of the excretory system could not be observed.

The testes are oval, elongate, the posterior testis about its length from the posterior end of the body. There may be a short interval between the testes or they may be almost contiguous. The posterior testis is slightly larger than the anterior one and measures from 0.56 to 0.65 mm. in length and 0.22 to 0.28 mm. in width. Sperm ducts are not visible in the whole mounts; in most of the specimens the cirrus-sac extends about the diameter of the acetabulum behind that sucker; in the one shown in Figure 16, the cirrus-sac is coiled and consequently does not extend as far posteriad. The posterior one-half to two-thirds of the cirrus-sac is filled with a seminal vesicle and the anterior portion contains the eversible male duct, surrounded by secretory cells. The cirrus bears small recurved spines and the genital pore is median at the anterior margin of the acetabulum.

The ovary is spherical, 0.18 to 0.20 mm. in diameter, situated a short distance in front of the anterior testis. The oviduct arises at the posterior margin and the ootype and Mehlis' gland are posterior to the ovary. The vitellaria are lateral to and somewhat overlap the digestive ceca dorsally and ventrally. The follicles are spherical to oval, 0.04 to 0.065 mm. in diameter, continuous on both sides of the body, although somewhat reduced in one specimen at the level of the posterior testis. They extend from the posterior end of the body about two-thirds of the distance to the anterior end, terminating about two-thirds of the distance from the ovary to the acetabulum. Transverse ducts at the level of the ootype pass mediad to form a vitelline receptacle which discharges into the oviduct immediately before the ootype. The initial portion of the uterus is filled with spermatozoa. The eggs are broadly oval, those near the ovary average 0.100 by 0.076 mm., those farther along in the uterus are rounded, often collapsed, and may be slightly longer.

Himasthla mcintoshi agrees most closely with *H. rhigedana*, type of genus. Both are from species of *Numenius* and they are the only described species with more than 31 spines on the collar. Dietz reported a total of 34 to 38 spines in *H. rhigedana*, with 2, 3, or 4 corner-spines at each end of the row. In both of these species the corner-spines are very close together and often superimposed on those of the lineal row. Contractions of muscles in these locations produce variable orientation of the spines, so determination of their number and disposition is difficult. In Figure T of Dietz, the upper corner-spine on the left side could be interpreted either as a corner-spine or as the terminal spine in the collar-row. The two species differ in geographical distribution; *H. rhigedana* is from Arabia and *H. mcintoshi* from northwestern United States. Although the suckers do not differ greatly in size, *H. rhigedana* is more than twice as large as *H. mcintoshi*, the reproductive organs are much larger, although the eggs are smaller. The most obvious difference is in the disposition of the vitellaria; in *H. rhigedana* the vitellaria are interrupted at the testicular levels whereas in *H. mcintoshi* the follicles are continuous.

The type specimen is deposited in the U. S. National Museum Helminthological Collection under number 54,721.

Himasthla piscicola n. sp.

This species is based on specimens found by Dr. H. O. Ewert, veterinarian of the Zoological Society, Toledo, Ohio, in the alimentary canal of a fish, *Arapaima gigas,* from the Amazon River, Brazil. They were sent for determination to Dr. Leonard Allison of the Institute for Fisheries Research at the State Fish Hatchery, Grayling, Michigan. With the specimens there was the following information: "HISTORY; the 24 inch specimen arrived here in September. The fish ate in the first weeks, four to five goldfishes three inches long, daily, and came gradually down to one fish a day until he stopped eating around the 18th of December. In this week he vomited several small balls of mucus. Under the microscope, these balls appeared to consist of cells, mucus and many flagellates, *Octomitus intestinalis.* AUTOPSY; the abdominal investigation showed inflammation of the intestinal tract as well as the abdominal lining (peritonitis). The stomach lining was highly inflamed and congested. The stomach cavity was filled with a tenacious mucus and a certain parasite, which will be found separated on the accompanying slide." According to Allison (*in litt.*), the parasite was a trematode which had been mounted in water under a cover-glass and arrived perfectly dry. Other specimens were removed from the contents of the stomach which was preserved in water. Allison identified the worms as members of the genus *Himasthla.* Professor S. Yamaguti examined certain of the specimens and agreed with the generic determination, noting differences between these specimens and *H. tensa* Linton, 1940. Subsequently, Allison wrote Dr. G. R. La Rue, at the Animal Parasite Research Laboratory, Beltsville, Maryland, and sent him two of the worms in the belief that La Rue would write the description. But Dr. La Rue suggested that the writer examine the specimens and make the report. They are here described as a new species, *Himasthla piscicola.*

The two specimens measure 8.2 and 9.8 mm., respectively, in length. The larger one, shown in Figure 15, is designated as type. The organs of the smaller worm are almost as large as those of the type specimen. In the smaller one there are masses of spermatozoa in the initial portion of the uterus but no eggs. There are two eggs, one of them collapsed, in the uterus of the larger worm. In these specimens the reniform collar, open ventrally, delimits a short, flattened area at the anterior end of the body. The collar-spines are intact but other spines have been lost. There are 29 spines on the collar, 25 in the lineal row and two on either side behind the terminal ones. Those at the ventral corners are as large as those in the lineal row; they measure 0.085 mm. in length and 0.025 in maximum width. Behind the collar there is a short, neck-like constriction. The specimens are much extended, a result of their protracted immersion in water, and the uterine region between the cirrus-sac and the ovary is especially narrow. In the larger worm the width at the collar is 0.875 mm. The acetabulum is 0.44 mm. long and 0.50 mm. wide; it is about its diameter behind the collar. The oral sucker protrudes slightly and measures 0.18 by 0.19 mm. The pharynx is large, 0.24 mm. long and 0.11 mm. wide. The esophagus extends to the level of the acetabulum and the digestive ceca end blindly near the posterior end of the body. The testes are situated much nearer the middle than the posterior end of the body.

They partially overlap, the posterior third of the anterior testis is in the same zone as the anterior third of the posterior testis. They are elongate, slightly notched; the anterior one is 0.875 mm. long and 0.20 mm. wide, the posterior one is 0.89 mm. long and 0.20 mm. wide. The cirrus-sac extends posterior to the acetabulum more than twice the diameter of that sucker. A large seminal vesicle occupies the posterior half of the sac; the anterior portion of the vesicle and the succeeding duct are enclosed in a large, many-celled prostate gland. The cirrus is not protruded and no spines were observed. The ovary is situated near the middle of the body, only a short distance in front of the anterior testis, but would be relatively more posteriad if the uterus were filled with eggs. It measures 0.18 by 0.20 mm. The ootype complex is large, situated immediately posterior to the ovary; the initial portion of the uterus is filled with spermatozoa and there are two eggs in the uterus. One is collapsed, the other measures 0.114 by 0.064 mm. The vitelline follicles are continuous on each side of the body and extend from the posterior ends of the digestive ceca about three-fourths of the distance from the ovary to the posterior end of the cirrus-sac. The follicles would probably extend farther forward in more mature individuals.

Although the specimens are not mature, *H. piscicola* differs from all other adequately described species with 29 collar-spines in the position of the gonads, the shape and overlapping arrangement of the testes, and in the relative length of the posttesticular region of the body. *Himasthla piscicola* and *H. annulata* were found in the digestive tract of fishes from the Amazon River; it is possible that the two are identical, that some avian species is the natural host, and that the discovery of these worms in fish hosts is entirely incidental. The worms are similar in size, but the description and figures of Diesing give no information concerning internal morphology of *H. annulata* and it is quite impossible to determine whether the two are identical.

The type specimen of *H. piscicola* is deposited in the Helminthological Collection of the U. S. National Museum under the number 39,445.

Discussion

An investigation conducted by the U. S. Bureau of Commercial Fisheries is attempting to determine the causes for the decline in populations of *Mya arenaria* along the coast of New England and possible biological measures for control of the principal predators, the green crab (*Carcinus maenas*) and the horseshoe crab (*Limulus polyphemus*). *Mya arenaria* harbors the sporocysts and cercariae of *Cercaria myae* Uzmann, 1952; the larval stages of an as yet undetermined species of *Gymnophallus* (Stunkard and Uzmann, 1958). The palps and gills contain metacercariae of digenetic trematodes. Since the asexual generations of these metacercariae must occur in mollusks which live in the immediate vicinity of the infected clams, a survey of the more abundant species and those most likely to carry the trematode infections, is in progress. Furthermore, since the definitive hosts of these metacercariae are animals that feed on *M. arenaria*, examination of shore-birds has been started. To obtain precise information under controlled conditions, metacercariae from *M. arenaria* have been fed to laboratory-reared eider ducks, herring gulls, common terns, white mice, and golden hamsters. The results, together with other pertinent information, are presented in this paper.

The metacercariae from *M. arenaria* proved to be larvae of three different species, all in the genus *Himasthla*, and the adults recovered from *L. argentatus* have been of value in resolving taxonomic problems in the genus. Typically, echinostomes are parasites of warm-blooded vertebrates and members of *Himasthla* have metacercarial stages in marine mollusks and mature in avian hosts. *Himasthla ambigua* Palombi, 1934 was described from metacercariae found in *Tapes decussatus* from the Gulf of Naples, but the adult stage is yet unknown. Palombi reported that the worms have 32 cephalic spines, that the infection is seasonal, and suggested that a bird, perhaps a migrant, is the final host. Elucidation of the life-cycle of *H. compacta* supports previous belief that species of *Himasthla* are parasites of birds and that the larvae occur in marine mollusks. Although adults of *H. compacta* are less than one-half the size of those of *H. quissetensis*, the cercariae of the two species are almost identical in size.

Since members of *Himasthla* typically are parasites of shore-birds, it is surprising that four species have been recorded from abnormal hosts, three from fishes and the fourth from a fruit-eating pigeon. Two species, *H. multilecithosa* from the pigeon and *H. piscicola* from *Arapaima gigas*, were taken from captive hosts and the time and place of infection are unknown. The finding of specimens of *Himasthla* in the digestive tract of marine and fresh-water fishes presents a biological anomaly. The fishes could have ingested a bird or its entrails that had fallen in the water and the proposed identity of *H. tensa* and *H. elongata* suggests such an explanation. *H. annulata* and *H. piscicola* were taken from the alimentary tract of fishes from the Amazon River of Brazil, and if the worms found in *A. gigas* were acquired in South America, they must have persisted for an unusually long time in the fish. The specimens were still juvenile, which indicates that they were recently ingested or that they fail to attain sexual maturity in the cold-blooded host. Since *H. annulata* (Diesing, 1850) and *H. piscicola* are from Amazonian fishes, it is possible that the two are identical, that some avian species is the natural host, and that the discovery of these worms in the digestive tract of fishes is entirely incidental.

SUMMARY

The validity of species in the genus *Himasthla* is discussed; *Echinostoma annulatum* (Diesing, 1850) is transferred to *Himasthla* and *H. tensa* Linton, 1940 is suppressed as a synonym of *H. elongata* (Mehlis, 1831). A specimen from *Nycticorax nycticorax*, tentatively assigned to *H. elongata*, is very similar to *H. secunda* (Nicoll, 1906), which suggests the possibility that *H. secunda* may be a not-fully mature form of *H. elongata*. Three new species are described; *H. mcintoshi* from *Numenius americanus americanus* taken in Tooele County, Utah; *H. piscicola*, probably an accidental infection, from the South American fish, *Arapaima gigas*; and *H. compacta* from experimental infection of the herring gull, *Larus argentatus*. The life cycle of *H. compacta* has been traced; the asexual generations occur in *Hydrobia minuta*, the cercariae encyst in *Mya arenaria* and probably other mollusks.

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THE SEASONAL OCCURRENCE OF MYTILUS EDULIS ON THE CAROLINA COAST AS A RESULT OF TRANSPORT AROUND CAPE HATTERAS[1]

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The edible mussel, *Mytilus edulis* L., attaches to rocks, pilings, and other firm substrates, being particularly abundant in the lower intertidal zone, where it is a dominant organism in the community (Newcombe, 1935; Dexter, 1947). A temporate and boreal species, it is found on both American and European coasts of the North Atlantic as well as Asian and American coasts of the North Pacific Ocean. Its distribution has aroused the interest of ecologists as an example of the effects of temperature on determining the geographical limits of species. Hutchins (1947) has suggested that the southern limit of *M. edulis* occurs where the mean sea surface temperature during the warmest month is approximately 80° F. (26.6° C.).

In the western Atlantic, *M. edulis* reaches its southern limit in the Carolinas. Although Hutchins showed the 80° summer isotherm intersecting the coast in the vicinity of Cape Hatteras, the edible mussel has been reported as far south as Charleston, South Carolina, three hundred miles below Cape Hatteras. This apparent contradiction of Hutchins' temperature limitation of *M. edulis* is discussed and related to the biology of the species. Its occurrence on the North Carolina coast is analyzed in relation to coastal water masses, their temperature and movement, and meteorological conditions.

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OBSERVATIONS

Mytilus edulis has been collected in the course of study of littoral organisms in the Beaufort and Cape Hatteras areas (Fig. 1). In the latter region, this study has centered on fauna attached to a group of eight wrecks near the outer beach. All but one wreck ordinarily extend above the water surface; all are metal and provide a firm substrate for many sessile organisms. *Mytilus edulis* occurs all year long on wrecks north of Cape Hatteras (stations 1–6: Kitty Hawk, Overlook, Pea Island, Rodanthe, Waves, and Salvo), where it is the dominant organism in the lower part of the intertidal zone and extends into deeper water on subtidal surfaces.

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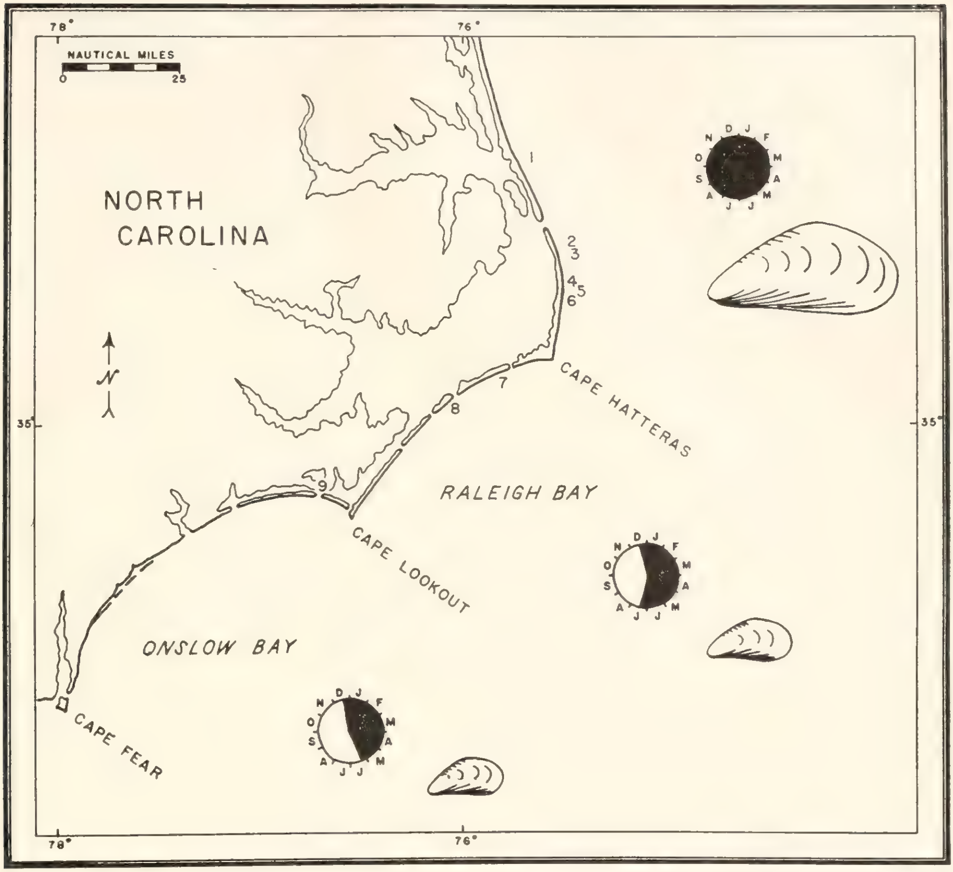

FIGURE 1. Chart of North Carolina coast, showing stations for the collection of *Mytilus edulis,* the portion of the year that *M. edulis* is present in each region, and the relative size attained by this species in each region. The blackened portion of the clock symbol indicates the presence of *M. edulis.* Station 1—Kitty Hawk, 2—Overlook, 3—Pea Island, 4—Rodanthe, 5—Waves, 6—Salvo, 7—Ocracoke Island, 8—Portsmouth Island, 9—Beaufort.

Here, it attains a maximum size of at least 75 mm. However, on the two wrecks in Raleigh Bay, south of Cape Hatteras (Ocracoke and Portsmouth, stations 7 and 8), *M. edulis* has been found only in spring, disappearing in late July. The maximum size attained in Raleigh Bay is 33 mm.

In the Beaufort area (station 9), *M. edulis* has been collected on rock jetties and pilings near Beaufort Inlet, where it shares its intertidal habitat with *Brachidontes exustus,* a small ribbed mussel which is distributed from North Carolina to the West Indies. Variable in abundance from year to year, *M. edulis* in the Beaufort area is often relatively scarce, with a few individuals scattered among large populations of *B. exustus.* Occasionally, however, it is very abundant in the spring, far outnumbering *Brachidontes.* While adequate records over a long period of time have not been kept, it is known that the appearance of *M. edulis* in large numbers occurs at irregular intervals. It was very abundant in June, 1938; Stephenson and Stephenson (1952) noted that it was common locally in April and May, 1947; and there have been several years in the past decade when it has been abundant in the spring. *M. edulis* appears during the winter months. Ordinarily it disappears in June, leaving *B. exustus* as the predominant mussel on pilings and jetties in the intertidal zone. In the Beaufort area, *M. edulis* is small, attaining a maximum size of only 30 mm.

While *M. edulis* is a permanent resident and a dominant organism of the intertidal zone north of Cape Hatteras, the niche it occupies is largely taken south of the Cape by *B. exustus*.

Discussion

Temperature

The differential success of *M. edulis* on the North Carolina coast can be attributed to differences in water temperatures. Two distinct types of water lie off the coast, exhibiting different temperature and salinity characteristics. These have been designated (Bumpus and Pierce, 1955) as Virginian Coastal water, extending northward from Cape Hatteras to Cape Cod, and Carolinian Coastal water, extending southward from Cape Hatteras to Cape Canaveral. Virginian Coastal water is much colder than that of the more southern Carolinian Coastal water (Fig. 2). At Cape Hatteras, there is typically a sharp temperature gradient (Parr, 1933), and these water types remain relatively distinct, with little mixing.

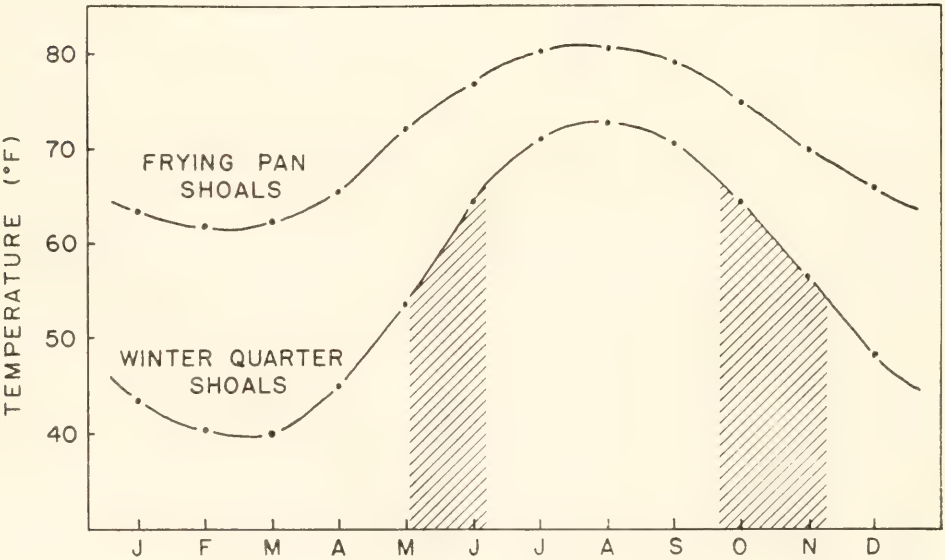

Figure 2. Monthly means of sea surface temperature at Frying Pan Shoals Lightship (Carolinian Coastal water) and Winter Quarter Shoals Lightship (Virginian Coastal water). Shading indicates periods of temperature suitable for attachment of *Mytilus edulis* in the southern part of Virginian subprovince. Data for 1947–1956 for Frying Pan Shoals and 1925–1940 for Winter Quarter Shoals from Bumpus (1957a).

Biologically, these temperature conditions are extremely important, determining which species survive along each part of the coastline. The temperature gradient in the Cape Hatteras area helps to separate the fauna of the Virginian biogeographic subprovince from that of the Carolinian subprovince. On the basis of their respective temperature regimes, the Virginian subprovince can be classified as a temperate zone (8–25° C., 46–77° F.), and the Carolinian subprovince can be classified as a sub-tropical zone (15 to almost 30° C., 59–85° F.) in the classification system of Vaughan (1940).

South of Cape Hatteras, summertime maximum temperatures may exceed the thermal limits of *Mytilus edulis*. Ritchie (1927) found that this species was killed by a fourteen-hour exposure to 84° F., and Bruce (1926) has indicated that it could not survive more than one hour at 86° F. We have recorded water temperatures as high as 84° F. along the shore of Raleigh Bay in August, 1959, and Maturo (1959) recorded a similar temperature in the Beaufort area in August, 1954. These are not temperatures of estuarine water that had warmed over shallow inshore areas; they represent oceanic conditions to which mussel populations would be exposed. These summertime temperatures are sufficiently high to cause the death of any edible mussels south of Cape Hatteras. Death in nature may precede the attainment of the high temperatures cited.

The annual date of disappearance from the natural habitat is advanced or delayed in relation to the progression of the temperature cycle. For example, in the spring of 1956 when mean air temperatures in eastern North Carolina were several degrees lower than average (U. S. Weather Bureau, 1956), and water temperatures along the middle Atlantic Coast were similarly depressed (Bumpus, 1957b), the delay in vernal warming permitted *Mytilus edulis* and many other organisms of the winter sequence to persist in the Beaufort area well into July. In the Beaufort area, the disappearance of mussels more closely coincides with the attainment of a water temperature of 80° F. It seems likely that higher air temperatures and solar radiation raise the temperature of mussels to a critical level during their exposure at low tides. Under these conditions, the 80° isotherm chosen by Hutchins to represent the southern limit of *M. edulis* serves better than 84° as an empirical expression of the ecological requirements of this species. The different dates of disappearance recorded for the Beaufort area (mid-June) and for the Ocracoke area (late July) reflect a later date for the attainment of lethal high temperatures in the more northern Ocracoke area.

North of Cape Hatteras, such high temperatures do not occur in the ocean, and *M. edulis* survives the maximum temperatures of summer. Just as an upwelling of cool bottom water permits *M. edulis* to live on the coast of Lower California, Mexico (Woods Hole Oceanographic Institution, 1952), an upwelling of cool subsurface water contributes to the maintenance of a suitable environment for this species north of Cape Hatteras (Wells and Gray, 1960). The occurrence of this cooler water north of the Cape permits the maintenance of sizable populations of other northern species with boreal affinities (Wells *et al.*, 1960).

Water movements

In view of the fact that Carolinian Coastal water is too warm to permit year-round survival south of Cape Hatteras, *Mytilus edulis* in this region must owe its existence to larvae originating from the extensive populations north of the Cape, which could renew colonies south of the Cape, if they were provided suitable transport. However, while it is recognized that a southward-flowing coastal current follows the edge of the continental shelf off the middle Atlantic Coast, this coastal current normally does not pass Cape Hatteras; instead, it turns seaward and parallels the Gulf Stream, flowing in a northeasterly direction (Bigelow, 1933; Ford *et al.,* 1952). The Gulf Stream System approaches Cape Hatteras from the south and then flows northeastward, away from the North American mainland (Iselin, 1936). Because the Outer Banks of North Carolina project across much of the continental shelf, the average position of the inner edge of the Gulf Stream lies only ten miles off the point of Cape Hatteras (Marshall, 1951), and the Gulf Stream System has a marked effect on this part of the coastline. As a result of the convergence of these two currents on the Cape Hatteras region, there is a pronounced offshore movement of water. Bumpus (1955) noted the offshore movement of several drift bottles released in 1950 in the Cape region. Such conditions would appear to bar the movement of mussel larvae around Cape Hatteras; instead, the larvae would be carried out to sea.

Although there is no regular movement of water around the Cape, Bumpus and Pierce (1955) witnessed the breaching of this barrier and postulated the sporadic occurrence of transient indrafts of Virginian Coastal water into Raleigh Bay, as the result of northeast storms in the Cape Hatteras region. The duration of a northeast storm and the subsequent weather pattern apparently would determine the fate of this parcel of Virginian Coastal water. If the storm lasts but one or two days, this water may eventually be absorbed within the Carolinian Coastal water by mixing, or a violent meander of the Gulf Stream may sweep over the continental shelf, pushing or drawing the Virginian Coastal water to the northeast. If the northeast storm persists or is followed closely by another northeast storm, the parcel of Virginian Coastal water may grow in size and be driven farther southward, around Cape Lookout into Onslow Bay. Northeast storms, common in fall and winter months, are a characteristic component of the climate on this coast. Strong northeasterly winds push water southward past the point of Cape Hatteras. By the mechanism described by Chase (1959) concerning the effect of wind on water movement, wind tides produced north of the Cape help to move Virginian Coastal water across Diamond Shoals.

Bumpus and Pierce (1955) found Virginian Coastal water in Raleigh Bay in January, 1954, after a three-day storm. A similar parcel of water with the characteristics of Virginian Coastal water was noted at Frying Pan Shoals Lightship in November, 1956, following an extended period of northeast storms in the Hatteras area (Bumpus, 1957b). Again, for April, 1958, Day (1960) recorded the occurrence of Virginian Coastal water at Frying Pan Shoals, 145 miles southwest of Cape Hatteras. Bumpus (1957b) noted that this southward movement of water had progressed at the rate of seven or eight miles per day. At the height of a storm, water has been observed flowing past the point of Cape Hatteras at a rate of one to two knots. After having paralleled the coastline north of the Cape, it continues to flow in a southward direction rather than following the coast westward.

Bumpus and Pierce suggested that this southward movement of water might distribute northern planktonic elements southward along the coast, and that this might account for the presence of anomalous species of northern affinities in the winter-spring sequence in Onslow Bay. Both Williams (1948, 1949) and Sutcliffe (1950) had found a number of northern species in this area whose occurrence was apparently restricted to the cold months of the year. *Mytilus edulis* appears to be such an anomalous species in the Carolinian subprovince. Larvae of this species from north of Cape Hatteras could be distributed along the coast south of the Cape by such a water movement.

Settlement period

To be effective in the transport of *Mytilus edulis* larvae, these storms must occur during the reproductive period of the mussel. The period of breeding and attachment of this species occurs at different times in different parts of its range, apparently in response to temperature conditions. Engle and Loosanoff (1944) found *M. edulis* settling in early June at Milford, Connecticut, when the water temperature was 12.5° C. (about 54° F.). Attachment reached a peak about a month later, then fell off when water temperatures reached 19° C. (66° F.). After a brief halt in the first half of August, some attachment occurred in late August, when water temperatures were declining from the seasonal maximum. A comparison of the dates of attachment from other studies (Table I) shows that attachment typically follows the pattern observed at Milford, beginning near 12° C. (54° F.) and reaching a maximum at 15 to 18° C. (59–64° F.).

TABLE I

Attachment of Mytilus edulis *at various locations*

Locality	Beginning		Maximum		Reference
	Date	Temp. (° C.)	Date	Temp. (° C.)	
Lamoine, Me.	June (late)	13	July (end)	15	Fuller, 1946
Milford, Conn.	June 1	12	June 15	15	Engle and Loosanoff, 1944
Oakland, Calif.	March	14	May	18	Graham and Gay, 1945
Kola Fjord, USSR	July	12	—	—	Zenkevitch, 1935
Millport and Caernarvon, U.K.	July	12	—	—	Bengough and Shepheard, 1943
Plymouth, U.K.	June	13	—	—	Mott, 1944
Kanazawa, Japan	January	10	May	17	Miyazaki, 1938

In the southern part of the Virginian subprovince, there appear to be two important periods of settlement of *Mytilus* larvae, one in late spring and one in late fall, well separated by a warm period when little or no settling occurs. Spring attachment may begin in April, when water temperatures warm to about 54° F., and extend into early June. Optimum conditions are again realized in October and November, when water temperatures are between 66 and 54° F. These temperature intervals are interposed on the graph of water temperatures at Winter

Quarter Lightship in Figure 2. Evidently, the late fall attachment period is more important than the late spring attachment period for colonizing the northern part of the Carolinian subprovince. Northeast storms are more likely in the October-November period than in late spring, and mussels which attach in May are soon killed by high temperatures south of the Cape. Only the late fall set would have the opportunity to grow to sizable dimensions. *Mytilus edulis* has been noted in the Beaufort area starting in December with representatives of the fall set. Only representatives of this set could reach a length of 30 mm. by the time the summer mortality sets in. Therefore, northeast storms in fall months should be primarily responsible for the appearance of mussel populations south of Cape Hatteras.

Correlation with weather records

From the above discussion, it would appear that the occurrence of *Mytilus edulis* south of Cape Hatteras can be directly related to movements of water around the Cape resulting from northeast storms. However, to be effective these storms must occur during the fall reproductive period for this species. An examination of the cycle of monthly mean water temperatures shows that this period ordinarily occurs in October and November (Fig. 2), but warmer than average conditions in September will extend the attachment period into December, and cooler than average conditions in September will make the latter part of that month suitable

TABLE II

Northeast storms in the Cape Hatteras area suitable for the transport of Mytilus edulis *larvae south of Cape Hatteras, 1954 to 1958, with relative abundance of* Mytilus edulis *at Beaufort the following spring*

	Number of days	Dates	Mean wind velocity (mph)	*Mytilus edulis*
1954	—	none	—	none
1955	3	October 2–4	14.0	rare
1956	7	October 10–16	17.7*	
	3	October 19-21	14.5	
	6	October 24–29	20.4*	abundant
	5	November 2–6	15.3	
	—			
	21			
1957	3	October 3–5	10.8	moderately abundant
	5	October 11–15	13.0	
	—			
	8			
1958	4	October 6–9	16.9	
	4	October 18–21	10.6*	abundant
	—			

* Severe storm; tides 2 to 3 feet above normal.

for attachment. Bumpus and Pierce noted that a short-lived storm is not effective in causing a significant penetration of water south of Cape Hatteras, and suggested that a storm that lasted for three days or more was necessary to produce the southward movement. With these criteria in mind, weather records for Cape Hatteras (Local Climatological Data) have been examined for the occurrence of northeast storms during the five-year period from 1954 to 1958. Only those storms were counted in which a northeasterly wind (NNE, NE, or ENE) predominated three or more days, with a mean velocity in excess of 10 mph. Using water temperature records by Bumpus (1957a, 1957b) and Day (1958, 1960) as a guide to the late fall attachment period of *M. edulis,* the northeast storms which could have carried mussel larvae south of Cape Hatteras during this five-year period are listed (Table II).

Mean wind velocity is included in Table II as a measure of the likely effectiveness of each storm in moving Virginian Coastal water southward. While 1958 storms extended over as many days as 1957 storms, a greater severity and effectiveness of the 1958 storms is indicated by a greater wind velocity. The fall of 1956 was distinguished by a remarkable series of storms in October and early November, with only brief intermissions. It was after these storms in 1956 that a parcel of Virginian Coastal water was detected off Frying Pan Shoals.

Relative abundance of *Mytilus edulis* at Beaufort the following June is also included in Table II. Although *M. edulis* may comprise up to 80% of the mussels on the jetties and pilings, such populations are uncommon. Ordinarily, it comprises less than 10% of the mussels, the great majority being *Brachidontes exustus.* If *Mytilus edulis* colonies in the Beaufort area owe their existence to a transport of larvae around Cape Hatteras by northeast storms the preceding fall, a close correlation would be expected between the number and severity of northeast storms and the abundance of *M. edulis* at Beaufort the following June. A comparison of these items in Table II shows a very close correlation. Not only does the occurrence of *M. edulis* agree quantitatively with the prior occurrence of northeast storms, but this species was not found in the spring following an exceptionally storm-free fall season in 1954. The greatest abundance of mussels was recorded after the remarkably stormy fall of 1956. The abundance of mussels also reflected the relative severity of storms in the fall of 1957 and 1958, mussels being more common after the 1958 season. It seems evident that this is a cause-effect relationship.

The presence of *Mytilus edulis* in the northern part of the Carolinian subprovince serves as an indication of the southward penetration of Virginian Coastal water. Consequently, the presence of *M. edulis* at Charleston, South Carolina, indicates that Virginian Coastal water may be driven this far south.

Other species

Other molluscs of northern affinities probably owe their sporadic appearance in the Beaufort area to a similar transport of their larvae southward around Cape Hatteras by northeast storms. For example, although the normal range of *Littorina saxatilis* in the western Atlantic extends from the Arctic to Cape May, New Jersey (Abbott, 1954), Stephenson and Stephenson (1952) reported that species in the Beaufort area during April and May, 1947. The anomalous appearance in the Beaufort area suggests that it, too, was transported southward around Cape Hatteras in larval stages. At Cape Ann, Massachusetts, Dexter (1947) noted spectacular fluctuations in abundance of *L. saxatilis* for year to year in the same location. Such variation in numbers indicates that larvae of this species are subject to water movements, and that they may be carried some distance from the parent colony. Its appearance in Beaufort would be in keeping with this type of distribution.

Summary

Although *Mytilus edulis* survives year-round north of Cape Hatteras, summer water temperatures are high enough to kill it south of that point. Populations of this species in the Carolinian subprovince are apparently maintained by an external source of larvae. Larvae can be transported around Cape Hatteras by southward movements of Virginian Coastal water that result from persistent strong northeast winds in late fall. The presence and abundance of *M. edulis* at Beaufort has been correlated with the abundance and severity of northeast storms occurring in the Cape Hatteras region during the preceding fall, over a five-year period. Probably other northern species owe their sporadic appearance in the Beaufort area to similar transport of larvae by southward water movements.

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THE INFLUENCE OF TEMPERATURE ON OSMOTIC REGULATION IN TWO SPECIES OF ESTUARINE SHRIMPS (PENAEUS)

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Estuarine decapod Crustacea living in an environment of varying salinities and temperatures face great osmoregulatory problems. Presumably such animals could either possess well-developed osmoregulatory powers or, lacking these, follow passively the fluctuations of the external medium. Migratory crustaceans present a special case, for their movements may carry them in and out of estuaries, often quite rapidly, thus complicating their osmoregulatory work.

Among swimming shrimps (Penaeidae) are many which live a wandering life, and on the southeastern and Gulf coasts of the United States three such species, *Penaeus setiferus* (Linn.), *P. duorarum* Burkenroad and *P. aztecus* Ives, occur in enormous numbers in and near estuaries.

The geographic ranges, life histories, migratory habits and general ecology of these species have been reported in whole or in part in numerous publications, including: Burkenroad (1934, 1939); Gunter (1950); Heegaard (1953); Lindner and Anderson (1956); Pearson (1939) and Williams (1955). It is well established that these species spawn at sea. The demersal eggs hatch into nauplii which undergo larval transformations while being transported shoreward; by the time they are transformed to the postlarval stage, they are entering estuaries where they proceed to shallow brackish areas to continue growth. With increasing age they move into deeper estuarine waters, return to sea with approaching sexual maturity, and seldom, if ever, return to the estuaries. The estuarine phase lasts about four months in summer but is extended in colder months.

Knowing this, the questions asked in this study are: do these animals regulate their internal salinities and, if so, how well do they accomplish osmoregulation in a variety of salinity and temperature combinations?

Similar questions have been posed by others. Panikkar (1941) found that *Leander serratus, L. squilla* and *Palaemonetes varians* in undiluted sea water are able to maintain their internal fluids at lower concentrations than the outside medium (hypotonic regulation), and in waters of low-salt content are able to maintain their internal fluids at higher concentrations than the outside medium (hypertonic regulation). Panikkar and Viswanathan (1948) found the same ability exhibited by *Metapenaeus monoceros* and Panikkar (1951) reported it shown to a lesser extent in *M. dobsoni, Penaeus indicus* and *P. carinatus* and in the grapsoid crabs *Eriocheir sinensis* and *Varuna litterata.* Panikkar (1951: page 171) states that this ability to regulate is "probably the most elaborate osmotic mechanism ever perfected by invertebrates" and is widely distributed among the

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Crustacea. Broekema (1941) and Verwey (1957) have shown that the blood of *Crangon crangon* is hypotonic in sea water and hypertonic in diluted sea water. Similar osmoregulatory patterns in a number of grapsoid and ocypodid crabs have been found by Edmonds (1935), Jones (1941), Gross (1955, 1957a) and in *Birgus latro* by Gross (1955).

The relationship of osmoregulation to temperature has not been so extensively investigated. Broekema (1941) found in *Crangon crangon* that young in summer tolerate lower salinities than adults. Adults seek higher salinities in cold weather and lower salinities in warm weather. The young, after a period of estuarine existence, go to sea in the fall and return to estuaries as adults the following summer, but they never again inhabit waters of as low salinity as they experienced when young. Broekema found that continued dilution of the external medium brought about continued dilution of the internal medium within limits, but in a constant salinity the concentration of blood increased with a fall in temperature.

Materials and Methods

Though three species of *Penaeus* are abundant in North Carolina, only two, *duorarum* and *aztecus,* are available in large numbers in nursery areas near Morehead City each year. These two species were used in this study. From spring to late summer *aztecus* is abundant on the nursery grounds and from midsummer to fall and winter *duorarum* is available. Because a wide range of sizes is represented in both species during the year and because shrimp of different sizes occupy different habitats, each species was divided arbitrarily into two groups, juveniles (under 100 mm. total length) and subadults (100 mm. and over). Only *aztecus* attained the larger sizes during the experimental period.

The problem of osmoregulation in these penaeids was approached as simply as possible. Freezing point depression (melting point) of blood was used as an indication of the ability of these animals to osmoregulate in various dilutions of sea water at different temperatures. Using modifications of the methods of Jones (1941), Gross (1954) and Hickman (1959) for determining melting points, small, uniform samples (approximately 1 mm.^{3}) of blood were taken in dried, thin-walled capillary tubes (1 mm. diameter), the ends of the tubes sealed with Vaseline, and the samples quick-frozen on dry ice. Samples were stored in a freezer until the melting points could be determined. Melting points of known standards of pure and salt water were determined with the aid of a differential thermometer.

Later, determinations of the unknowns were made by timing the end points of melting of the frozen crystals in the unknowns and standards as they warmed slowly in a waterproof, insulated box filled with ice brine, and by converting the melting times to degrees C.

Samples of blood were taken from individual shrimp by puncturing the sternum of the first abdominal segment with a pin or sharp awl, then catching the fluid in a capillary tube. Prior to making the puncture, the sternum and adjacent structures were thoroughly dried with absorbent cotton swabs. In large individuals, 70 mm. and above, fluid welled out and entered easily into the tubes by capillarity. In younger individuals the amount of fluid was often so small that it had to be sucked into drawn capillary tubes with the aid of a vaccine bulb. Most shrimp stood this treatment well (though a few small individuals were lost) and the wounds closed readily.

Experimentation was limited to juvenile and subadult shrimp, and experimental conditions for salinity and temperature approximated those found in the sounds seasonally, where salinity ranges from about 7‰ to 34‰ and temperature from 4° to 30° C.

Five series of round, wide-mouthed, five-gallon jars were used as aquaria. Each series consisted of five jars containing salinities of approximately 10, 15, 20, 25 and 30‰ sea water (hydrometer readings). Dilutions were made with tap water. Water was aerated for 24 hours at room temperature before animals were introduced. At the beginning of an experiment shrimp were transferred from outside holding tanks supplied by a running sea water system to the jars, four individuals per jar. (The system pumps 30–34‰ Sound water through hard rubber and polyethylene pipes.) Shrimp in almost all cases were caught a day or two before each experiment began. Prior to each experiment samples of water and blood from shrimp were taken from the outside holding tanks. One blood sample was taken at random from shrimp in each jar after shrimp were placed in jars for two to three hours at room temperature (25–32° C.). Shrimp sampled were returned to the jars. (The few specimens badly injured or killed in the initial sampling were discarded and replaced.) At this same time a water sample in a capillary tube was taken from each jar as a check on the salinity mixtures. After samples were taken, four series were subjected to gradually lowered temperatures in a cold room. The fifth series was maintained as a control at room temperature throughout the experimental period. In 48 hours the temperature was lowered from about 28° to 18° C. and another round of blood samples was taken; in 96 hours at 8° C. another round was taken. At the end of 96 hours all animals were discarded, the water thrown out, new mixtures prepared, and fresh shrimp used for replications. Animals were not fed during the experimental periods.

A number of difficulties were experienced with the technique described above. It is desirable to have the brine solution warm slowly, one degree C. per half hour (Gross, personal communication). I had trouble maintaining this rate of warming, even when the box was precooled, for on hot humid days (temperature near 32° C. and humidity near saturation) the box would sometimes warm at a rate of one degree in fifteen minutes. Securing samples of uniform volume in the initial round of samples each week in this climate in summer was difficult. At temperatures above 22° C. shrimp blood coagulates rapidly, almost on contact with glass. The initial blood samples were taken from animals in air temperatures of 27°–32° C. and extreme difficulty was experienced in securing unclotted and uniform samples. As a corrective measure initial sampling was done in the cold room which had been quickly cooled to air temperature of 18° to 22° C. (water in the aquaria remained at approximately 28° C.). This method helped but still the amount of clotting hindered taking samples of uniform volume. This lack of uniformity in sample size gave somewhat distorted results in terms of melting points, but replications smoothed the inequalities. The 48- and 96-hour samples were readily taken at the lower temperatures.

For the experimental series, replications of individual determinations for both blood and water were averaged (Table I). The average values were subjected to standard methods for linear regression analysis (Snedecor, 1956) in which melting point of blood was plotted against melting point of water (Figs. 2, 3 and 4). Each of the curves if extended to lower and higher salinities would probably assume a more or less sigmoid shape (Jones, 1941; Verwey, 1957), but in other crustaceans previously studied a considerable portion of the curve in the salinity range treated here, 10‰–30‰, is essentially a straight line. In this investigation a linear relationship for salt concentration of blood in this range of salinities is assumed.

Regression coefficients were subjected to analyses of variance and all except one were found significant at the 5% level. The one exception (*duorarum* experimental and control at 28.4° C.) which falls slightly short of this level of significance is attributed to sampling difficulty. If all regression coefficients are considered significant, an important question remains as to whether or not the lines in each figure

TABLE I

The number of determinations and their average values for the blood melting points shown in Figures 2, 3 and 4

		Mean salinity in ‰				
		10.06	15.11	20.17	25.09	30.50
P. aztecus 42–100 mm.						
Exp. & Contr. 28.3° C.	N Determinations	21	23	22	22	23
	$\bar{x}$ Melting point ° C.	−1.21	−1.33	−1.48	−1.56	−1.52
Exp. 48 Hrs. 16.2° C.	N	17	20	20	20	18
	$\bar{x}$	−1.10	−1.29	−1.34	−1.46	−1.57
Contr. 48 Hrs. 28.6° C.	N	4	3	4	4	4
	$\bar{x}$	−1.04	−1.26	−1.45	−1.56	−1.52
Exp. 96 Hrs. 8.8° C.	N	8	18	19	19	20
	$\bar{x}$	−1.13	−1.29	−1.41	−1.53	−1.75
Contr. 96 Hrs. 28.8° C.	N	5	6	6	5	6
	$\bar{x}$	−1.23	−1.33	−1.46	−1.45	−1.56
P. aztecus 120–150 mm.						
Exp. & Contr. 28.3° C.	N	10	10	9	9	8
	$\bar{x}$	−1.36	−1.44	−1.57	−1.69	−1.76
Exp. 48 Hrs. 16.2° C.	N	5	7	8	8	8
	$\bar{x}$	−1.22	−1.26	−1.41	−1.48	−1.58
Exp. 96 Hrs. 8.8° C.	N	2	4	7	7	8
	$\bar{x}$	−0.99	−1.24	−1.34	−1.56	−1.77
P. duorarum 35–100 mm.						
Exp. & Contr. 28.4° C.	N	24	26	26	26	26
	$\bar{x}$	−1.48	−1.45	−1.47	−1.54	−1.65
Exp. 48 Hrs. 17.8° C.	N	22	24	23	24	21
	$\bar{x}$	−1.32	−1.35	−1.47	−1.59	−1.65
Contr. 48 Hrs. 28.4° C.	N	6	6	5	6	6
	$\bar{x}$	−1.45	−1.51	−1.49	−1.62	−1.65
Exp. 96 Hrs. 8.75° C.	N	24	24	24	23	24
	$\bar{x}$	−1.27	−1.32	−1.45	−1.64	−1.74
Contr. 96 Hrs. 28.1° C.	N	6	6	6	6	6
	$\bar{x}$	−1.45	−1.50	−1.49	−1.62	−1.65

are essentially alike, and analyses of covariance (Snedecor, 1956) reported below were used to facilitate these comparisons.

There are at least three confounding factors in the preceding methods. The first two, non-uniform samples and variable warming rate of the brine solution, have been mentioned but the third requires some discussion. The source of dilutant was tap water rather than distilled water because the supply of distilled water was limited. This choice was made deliberately, in full knowledge that the tap water was hard. Salinities of the mixtures were determined by hydrometer, but because many mixtures were measured and averaged in determining the results, the readings were very close to titration values. As an added check, however, samples of the mixtures were taken in capillary tubes and determined by melting point methods, and here departure from expected values became apparent. The greater the dilution the more the melting point was depressed.

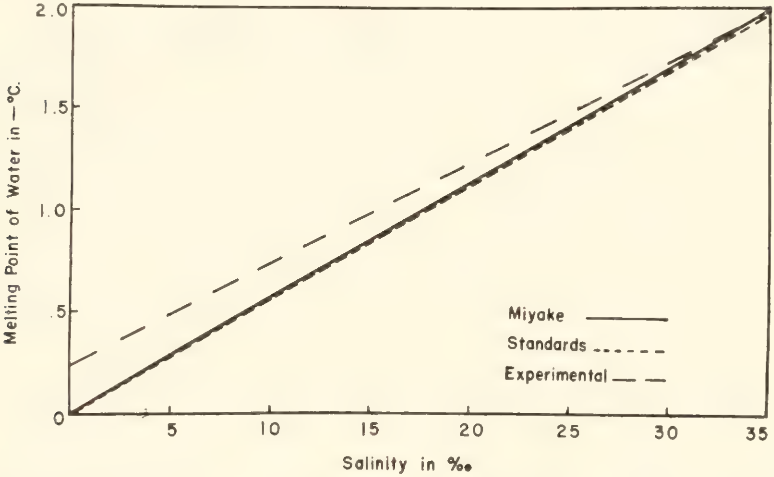

FIGURE 1. Comparison of the relationship between salinity and freezing point depression found by Miyake, that obtained for the standards used in these experiments, and that found in the experimental mixtures of tap water and sea water at average hydrometer-established salinities of 10.06, 15.11, 20.17, 25.09 and 30.50‰.

To determine exactly where the disparity in results lay, the values of Miyake (1939) were used as a basis for reference (Fig. 1). Next to the line for Miyake's values for salinity of west Pacific waters is a line formed by standards mixed for the series of experiments as determined by the differential thermometer. For all practical purposes the lines are identical. A line drawn through mean melting-point values for mixed salinities, however, does not coincide with the first two lines and indeed the deviation appears to be directly proportional to the amount of tap water used in dilution. This disparity is not deemed great and the melting points on the abscissa of Figures 2, 3 and 4 are those determined experimentally and shown in Figure 1. The effect of tap water on osmoregulatory behavior of the shrimp remains unknown.

RESULTS

Juvenile and subadult *aztecus* and *duorarum* are hypotonic to sea water in a salinity range of 30–34‰ in summer in a temperature range of 23–31° C. This was shown by a comparison of the melting points of blood from shrimps and water taken from outside holding tanks at the inception of each weekly experiment. This finding is in accord with the account for a number of decapod crustaceans (*supra cit.*).

Results of six weekly experiments on *aztecus* ranging in total length from 42–100 mm. are shown in Figure 2. Though shrimp in high salinity water prior to experiment are hypotonic, they are hypertonic to hypotonic in the array 10–30‰ after 2–3 hours of immersion in the mixtures at a mean room temperature of 28.3° C., those in the low salinities having more dilute blood than those in high salinities, for the line is not horizontal (b = .347). At the end of 48 hours, with an accompanying drop in temperature to 16.2° C., essentially the same relationship exists though blood in low salinities is even more dilute (b = .464). Controls at room temperature 28.6° C. show a somewhat similar picture (b = .512). At the end of 96 hours

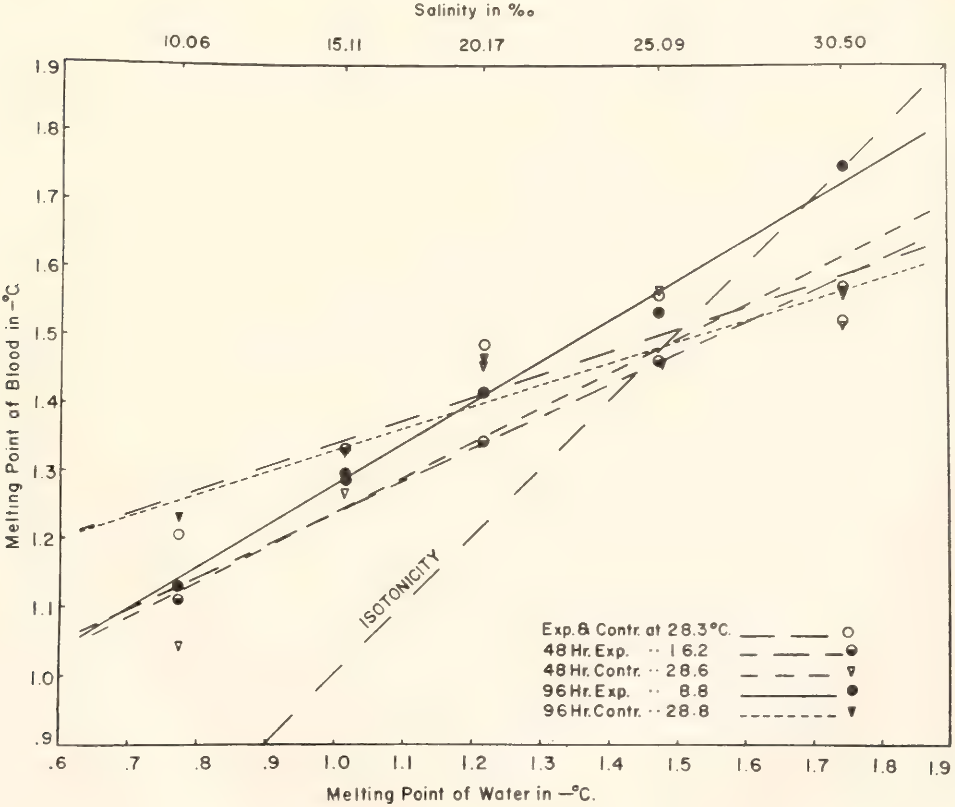

FIGURE 2. *Penaeus aztecus*, 42–100 mm. total length. Regression lines drawn through average melting points of blood at average melting points of water of −0.77, −1.01, −1.22, −1.47 and −1.74° C. Key to lines on figure.

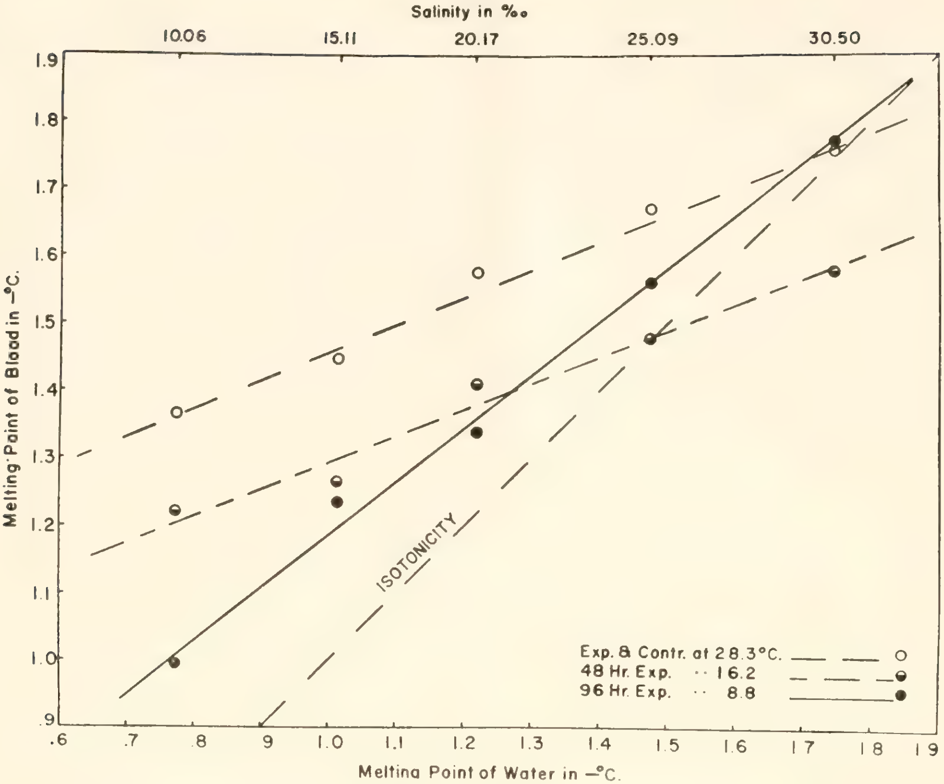

FIGURE 3. *Penaeus aztecus,* 120–150 mm. total length. Regression lines drawn through average melting points of blood at average melting points of water of −0.77, −1.01, −1.22, −1.47 and −1.74° C. Key to lines on figure.

at 8.8° C. the slope of the line has changed markedly (b = .613), the low values are nearly the same as those at 48 hours but values in the upper salinities have raised. The slope of the line indicates a trend toward isotonicity. At the same time the controls in room temperature, 28.8° C., have returned essentially to the same point at which they started (b = .323).

Analyses of covariance of data for initial experimental and control animals paired with 48-hour controls, 96-hour controls and experimental animals at 16.2° C. show no significant difference in slope or elevation ($P = 0.05$), but the line for animals in 8.8° C. water differs significantly in slope.

In larger *aztecus* (Fig. 3), essentially the same circumstances prevail though in accented form. The animals hypotonic to the sea water from which they were taken show a quick response to lowered salinities at room temperature (b = .427). At 48 hours with temperature lowered to 16.2° C. the slope of the line is approximately the same as in the beginning but the concentration of all bloods has been lowered (b = .385). At 96 hours the approach of the slope of blood salinities to the line of isotonicity is even more marked than in the experiments with younger *aztecus* (b = .783).

These lines represent averages from only two 96-hour runs; hence less reliance can be placed on them than those of the first experiment and, moreover, there are no controls with which to compare the results. The experiments were done in the hotter weeks of summer when aeration of aquaria was essential, especially when the jars held large animals. During one of the experiments the air-pump for controls stopped and during the other heat was above 30° C. In both cases distress and death among controls was too great to give valid results.

Analyses of covariance of the data for initial experimental and control animals paired with experimental animals at 16.2° C. shows that the slopes are parallel but the elevations differ significantly, and the line for animals in 8.8° C. water differs significantly in slope ($P = 0.05$).

Results of six weekly experiments on *duorarum*, 35–100 mm. total length, are shown as a series of regression lines in Figure 4. Again, animals hypotonic to the external medium at ambient temperature of 28.4° C. quickly make an adjustment

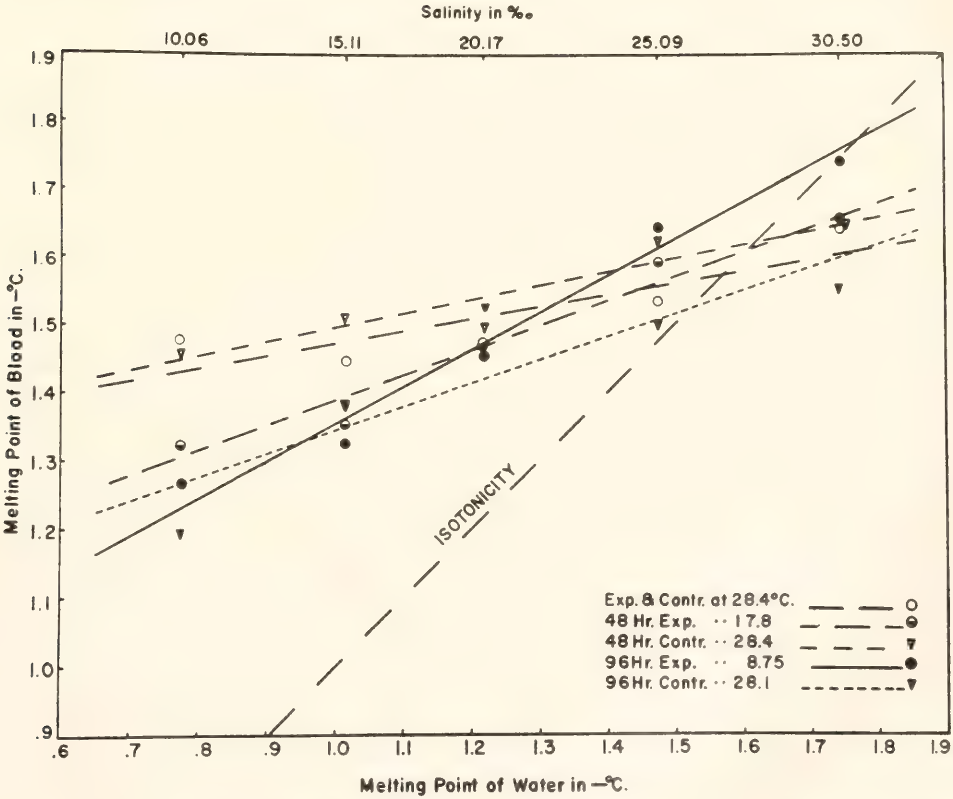

FIGURE 4. *Penaeus duorarum*, 35–100 mm. total length. Regression lines drawn through average melting points of blood at average melting points of water of −0.77, −1.01, −1.22, −1.47 and −1.74° C. Key to lines on figure.

when immersed in an array of 10–30‰ sea water mixtures (b = .182). Within three hours those in dilute media have more dilute blood than those in the less dilute. At the 48-hour point, with temperature gradually depressed to 17.8° C., the slope of this line has changed materially (b = .376). Though animals in 20‰ water have changed little, those in lower salinities have more dilute blood than in the beginning and those in high concentrations have more concentrated blood. Controls at 28.4° C. are essentially unchanged from conditions at the start, but those in higher salinities have somewhat more concentrated blood (b = .210). By the end of 96 hours at a temperature of 8.75° C. a marked change in the slope of the line for blood concentrations is again seen, with a trend toward isotonicity (b = .525). This trend is not so pronounced as in *aztecus* of comparable size (Fig. 2). In controls at 28.1° C. there has been a material dilution of blood at lower salinities (b = .343).

Table II

Per cent survival of shrimp at 48 and 96 hours. Forty-eight and 96 hours indicated as mean temperatures for those points. Significant figures in columns for Chi-squares are marked with an asterisk

				Per cent survival						
				Salinity					χ_a^2	χ_b^2
				10 ‰	15 ‰	20 ‰	25 ‰	30 ‰		
P. aztecus	Exp.	48 Hrs.	16.2° C.	58.8	83.6	90.0	92.5	88.8	17.98*	4.37
42–100 mm.		96	8.8°	37.5	77.5	85.0	87.5	80.0	41.55*	10.30*
	Contr.	48	28.6°	70.8	79.2	95.8	91.7	100.0	3.29	1.25
		96	28.8°	45.8	66.6	70.8	83.3	95.8	12.46*	5.42
P. aztecus	Exp.	48	16.2°	28.6	64.3	92.9	96.4	96.4	18.08*	3.79
120–150 mm.		96	8.8°	7.1	46.4	89.3	78.6	92.9	38.11*	13.97*
P. duorarum	Exp.	48	17.8°	67.7	92.5	98.9	98.9	97.8	10.27*	0.59
35–100 mm.		96	8.75°	63.4	89.2	97.8	94.6	96.7	13.82*	1.39
	Contr.	48	28.4°	66.7	86.4	91.7	100.0	100.0	3.88	1.21
		96	28.1°	62.5	77.3	91.7	95.8	100.0	5.63	2.25

Analyses of covariance of the data for initial experimental and control animals paired with 48-hour controls and 96-hour controls show no significant difference in slope or elevation ($P = 0.05$); however, the lines representing data for experimental animals at 17.8° C. and 8.75° C. both differ significantly in slope from the line representing the initial animals ($P = 0.05$).

It is instructive to compare survival of shrimp in the array of salinities and temperatures with the results of melting point determinations as expressed in regression lines (Table II, Figs. 2, 3, 4). Some deaths were due to injury, but if we examine the percentages (Table II) it is apparent that poorest survival was in 10‰ salinity. Chi-square (χ_a^2) values for differences in survival among salinities are significant in seven of the ten series ($P = 0.05$), indicating a high influence of salinity on survival. However, if we eliminate the animals so obviously affected by

10‰ salinity from the Chi-square totals (χ_b^2), it is apparent that higher salinities have little effect on survival. The only significant (χ_b^2) values ($P = 0.05$) are for *aztecus* young and adults at 96 hours in 8.8° C. water. This indicates a response to temperature such as suggested by the regression lines in Figures 2 and 3. Further, it is apparent that survival of *duorarum* is on the whole better than young *aztecus,* indicating greater tolerance to lowered salinity and temperature.

Discussion

Melting point determinations show a high degree of individual variation, as has been noted by Gross (1957b) for *Pachygrapsus crassipes.* Such variations may be attributed to stage in the molting cycle, age, sex, and in the present case perhaps to changes in salinity and non-uniformity of sample size. Replications tend to smooth these inequalities.

Each regression coefficient, except one, is significant at the 5% level. The exception is that for *P. duorarum* experimental and control at 28.4° C. and this instance nearly attains significance at the 5% level. The reason for this exception remains unexplained, though it is thought that the average blood melting point value of − 1.48° C. in water with a melting point of − 0.77° C. is low and perhaps not a good approximation.

Both of these species demonstrate possession of osmoregulatory powers. It is apparent that in the size ranges studied both species are hypotonic to sea water, but that in water under 30‰ they are hypertonic. This is in accord with Verwey (1957), who has suggested that it may be a general rule that Crustacea which regulate their internal environments are hypertonic in water of low salinity and hypotonic in water of high salinity.

Under the experimental conditions imposed, both species maintained themselves fairly well for limited periods in a range of 10–30‰ sea water, but with the lowering of temperature the regulatory powers meet more resistance. In such circumstances there appears to be a species difference. Juveniles and subadults of *P. aztecus* demonstrate a loss of osmoregulatory ability with lowering temperature which is statistically demonstrable after 96 hours, though a trend toward lowered activity is apparent even at 48 hours. In *duorarum* a significant difference was manifest in both 48- and 96-hour experimentally cooled samples. From the standpoint of per cent survival, though, indications are that *P. aztecus,* normally only a summer resident in North Carolina, does not regulate in lowered salinities at lowered temperatures as well as does *P. duorarum,* which is normally resident in North Carolina in winter in the juvenile state. The results provide experimental confirmation of information gained from field observations.

The results do not corroborate those of Broekema (1941) that salinity of blood increases with a fall in temperature, with the exception of shrimp in the 30‰ water, but it is possible that longer exposure to lowered temperature in a constant salinity might give different results. Likewise, the results are not in accord with those of Verwey (1957) that though an animal may not maintain a constant internal environment, it does tend to maintain a constant difference between internal and external environment in terms of osmotic pressure expressed in atmospheres at different temperatures. Again, longer exposure to a given temperature might alter this picture.

In both species it is shown that in all salinities the blood tends to approach isotonicity with the surrounding medium as temperature is lowered.

SUMMARY

1. Melting point determinations on blood of two species of shrimp, *Penaeus aztecus* and *P. duorarum*, were made in an array of salinities of 10.06, 15.11, 20.17, 25.09, and 30.50‰ at 28.1–28.8, 16.2–17.8 and 8.75–8.8° C.

2. These shrimp are hypotonic to sea water at room temperature and hypertonic to dilutions of sea water below 30‰.

3. These shrimp regulate moderately well in experimental dilutions at room temperature, though the blood is diluted somewhat in lowered salinities.

4. At lowered temperatures, 8.75–8.8° C., regulatory ability is impaired and blood tends toward isotonicity.

5. *P. duorarum* is a better regulator at low temperatures than *P. aztecus*.

6. Survival of these shrimp is better in higher salinities at low temperatures.

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